Management of Cylindrocarpon Black Foot Disease

in New Zealand nurseries and vineyards

A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy (Plant Pathology)

by

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Black foot disease of grapevines, a significant cause of vine death in New Zealand and world-wide, is difficult to control in nurseries and vineyards. This research investigated effects of fungicides, hot water treatment (HWT) and mustard biofumigation to control Cylindrocarpon black foot disease. These methods were tested against nine isolates, three each of the three *Cylindrocarpon* species; *C. destructans* (*Ilyonectria radicicola* species complex), *C. liriodendri* and *C. macrodidymum* (*Ilyonectria macrodidyma* complex), isolated from symptomatic grapevines from New Zealand vineyards and nurseries, *in vitro* and *in vivo*.

The *in vitro* testing of 12 fungicides showed that captan, carbendazim and didecyldimethyl-ammonium chlorine effectively inhibited conidium germination and mycelium growth of all nine isolates with EC_{50} values of 1.0-150, 0.05-100 and 1-1000 (mg a.i./L), respectively. Cyprodinil + fludioxonil, fludioxonil and tebuconazole were similarly effective at inhibiting mycelium growth ($P \leq 0.001$) and conidium germination ($P \leq 0.001$) of most isolates.

In two field sites, the fungicides significantly reduced disease severity ($P \leq 0.001$) and incidence ($P \leq 0.001$) in rootstocks 101-14 and 5C, with captan and carbendazim + flusilazole being effective in Auckland and cyprodinil + fludioxonil and Tricho-Flow™ effective in Blenheim. These products could therefore be used as preplanting dips and didecyldimethyl-ammonium chlorine (a sanitiser), could be used in nursery systems for prophylactic control of black foot pathogens on propagation material. Also tested, HWT (47°C for 30 min), was the most effective treatment against the black foot pathogens at both sites ($P \leq 0.001$).

The *in vitro* testing of different HWT protocols on mycelium growth, conidium germination and mycelium within grapevine canes showed that conidium germination was completely inhibited ($P < 0.001$) by heat treatments greater than 40°C for 5 min, and that mycelium growth was inhibited ($P \leq 0.003$) for all but one isolate by treatments greater than 47°C for 30 min. Within cane pieces, all *Cylindrocarpon* isolates were inhibited ($P < 0.001$; 0.0 to 0.9%

incidences) by 30 min at 48.5 and 50°C. In field trials, HWT protocols of 48.5 and 50°C for 30 min significantly reduced disease severity (P≤0.001) and incidence (P≤0.001) to 0%. This confirmed that HWT of 48.5°C for 30 min could replace the industry standard HWT of 50°C for 30 min to eliminate black foot disease in nursery grapevines in New Zealand.

Physiological effects on bud development and carbohydrate concentrations were examined after HWT, cold storage and different dates of harvesting dormant plants. HWT reduced disease incidence (P≤0.010) and severity (P≤0.001) as did later (July) harvest dates (P=0.010 and P=0.002, respectively), although cold storage had no significant effect (P>0.05) on disease levels. Over the months of harvest, root sugar concentrations were relatively constant (P=0.023) whereas starch levels were reduced (P<0.001). Cold storage increased sugar (P≤0.001) and reduced starch concentrations (P≤0.001) although the effect was less for HWT plants. Growth stages E-L 4 and E-L 9 were reached more slowly with plants harvested earliest and more quickly for HWT and cold stored plants (all P<0.001). Variety effects (P<0.001) showed that grafted grapevines 101-14 cv. Pinot noir reached growth stage E-L 9 most quickly, followed by 5C cv. Sauvignon blanc and then 101-14 cv. Sauvignon blanc. Results showed that if grapevines are not harvested in deep dormancy, cold storage for four weeks prior to HWT could improve rate and uniformity of bud development and reduce disease incidence.

In vitro biofumigation with *Brassica juncea* inhibited conidial germination and colony development, and mycelium growth of all *Cylindrocarpon* isolates (all P<0.001).
In field trials, mustard seed meal and plant material incorporated into the soil reduced disease incidence and severity in grapevine cuttings by 36 and 27%, respectively but not significantly (P= 0.359 and P=0.453). Despite the lack of statistical significance the experiments showed the potential of mustard biofumigation as a sustainable solution to nursery propagators or vineyard owners to control black foot disease.

These control strategies can reduce soil inoculum levels, protect young plants from infection and eliminate the pathogen from within plants, providing grape growers and nursery propagators with more tools for developing integrated and sustainable control systems.

Keywords: *C. destructans, C. liriodendri*, *C. macrodidymum*, hot water treatment, biofumigation, fungicides, disease incidence and severity, bud break, starch, sugar.

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"If we are facing in the right direction, all we have to do is keep on walking."

Buddhist Proverb

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Chapter 1

Introduction

1.1 History of wine grape production

The wine grape *Vitis vinifera* is thought to have originated in the temperate, climatic regions of the Caucasus in Asia. Grape growing and wine making spread to Asia Minor and into the Mediterranean and European countries; in Italy *V. vinifera* was even considered a native plant (Jackson and Schuster, 2001). With the development of the 'New World', grapevines and wine-making also spread with the European colonists into temperate regions, including North and South America, South Africa, Australia and New Zealand. Grapes were first grown in New Zealand by French settlers and religious missions in the early part of the 19th century (Jackson and Schuster, 2001).

Climate is one of the main factors determining where grapes can be grown, and the variety and quality of the wine produced. The most suitable latitudes for grape growing are between 30 and 51 degrees north, and 28 and 46 degrees south; virtually all quality wine is produced in regions situated in the temperate zones which have annual mean temperatures of between 10 and 20°C (Jackson and Schuster, 2001). Grapevines grow and yield poorly in regions where winters are too mild for dormancy to occur, and where summers are short and winters severe, crops may have insufficient time to ripen and cold winters can kill or seriously damage plants (Jackson and Schuster, 2001)

1.2 New Zealand wine industry

In New Zealand, wine grapes are largely produced in ten major wine growing regions that span latitudes 36° to 45° south and extending 1,600 km. They are, from north to south Northland, Auckland, Waikato/Bay of Plenty, Gisborne, Hawke's Bay, Wellington/Wairarapa, Nelson, Marlborough, Canterbury/Waipara and Central Otago (Smith, 2010). The major grape varieties grown are Sauvignon blanc, Pinot noir and Chardonnay, which occupy 48, 16 and 13%, respectively of the viticultural land. The grape and wine industry in New Zealand has expanded significantly over the past decade (Figure 1.1) from 10,197 ha in 2000 to

33,400 ha in 2012 (Smith, 2012). In 2012, New Zealand produced just under 2 million hectolitres of wine from which 1.78 hectolitres was exported at a value of $1.18 billion.

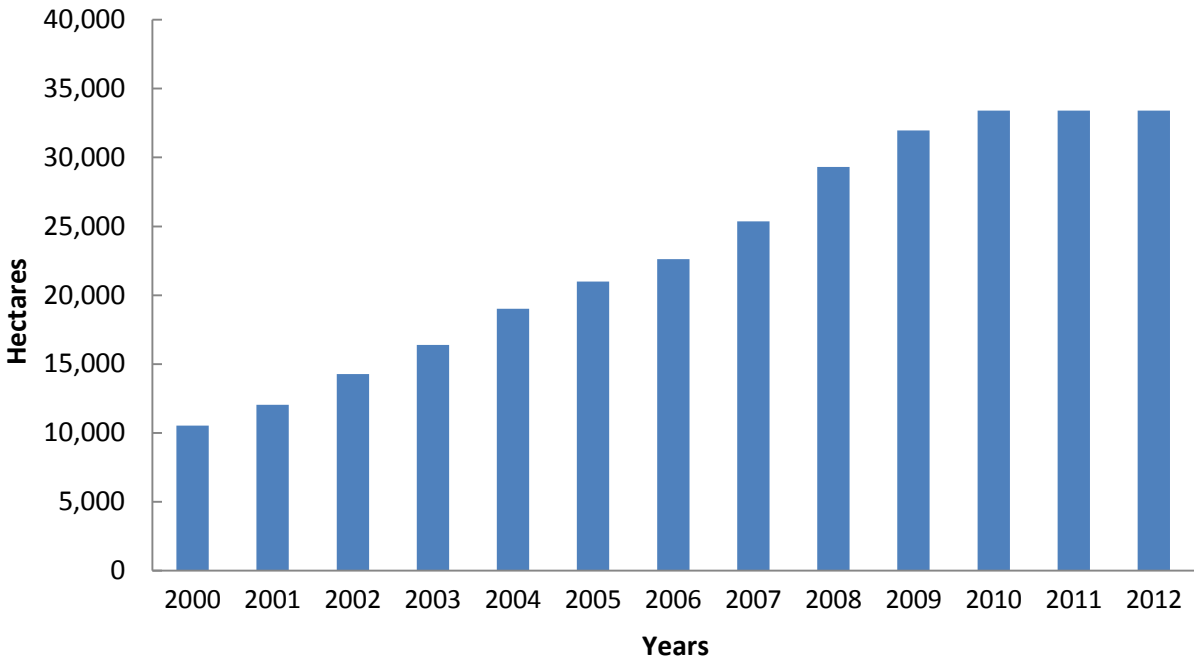


Figure 1.1 Wine producing area (ha) in New Zealand from 2000 to 2012.

1.3 Trunk Diseases of grapevines

In the last century the root-damaging soil-borne insect, *Daktulosphaera vitifoliae* (Fitch; Hemiptera: Aphididae) has spread into many grape-growing countries, in which it caused a problem called 'phylloxera'. The first reports of phylloxera in New Zealand vineyards were made in the Auckland district in 1885 and in 1900 the pest was discovered in Hawke's Bay (Gear, 1978), eventually spreading to the major grape growing regions of New Zealand. In 1980 the Ministry of Agriculture and Fisheries reported that of the 4800 ha of grapes planted in New Zealand, 90 percent of the area was planted with *V. vinifera* and 92 percent of these were planted on their own roots. With over 50 percent of these vines aged 2 years or less the potential damage by phylloxera imposed a serious problem to the New Zealand industry (King *et al.*, 1982). To combat the root damage caused, *V. vinifera* scion varieties were grafted during propagation to phylloxera resistant rootstocks, which usually comprised American *Vitis* spp. or hybrids of them. Since use of grafted grapevines became common,

there have been increasing problems with trunk diseases (Gubler *et al.*, 2004). These diseases have had significant economic effects due to lost production and replanting.

1.4 Cylindrocarpon black foot disease

In recent years, vineyards throughout the world have reported increasing numbers of declining young grapevines, especially in newly established and replanted vineyard sites (Oliveira *et al.*, 2004). The decline symptoms usually included stunting, chlorosis and late bud break that might be followed by death of the affected vines. Two diseases, 'Petri vine decline' caused by one or more species of *Phaeoacremonium* and *Phaeomoniella chlamydospora* (W. Gams *et al*.) Crous & W. Gams (Gubler *et al.*, 2004) and 'Cylindrocarpon black foot', caused by species of *Cylindrocarpon*, Wollenw., have been recognized as the major causes of this decline (Halleen *et al.*, 2005; Oliveira *et al.*, 2004). Black foot is generally believed to be the more serious disease of *V. vinifera* and non *V. vinifera* rootstocks as it frequently kills young vines within 1-2 years of planting out in the vineyard. It was first reported in France in 1961 (Maluta and Larignon, 1991) and has now been identified in all major viticulture regions throughout the world including: Italy (Grasso, 1984; Grasso and Magnano Di San Lio, 1975), California (Petit and Gubler, 2005; Scheck *et al.*, 1998), Portugal (Rego *et al.*, 2000), South Africa (Fourie *et al.*, 2000), New Zealand (Halleen *et al.*, 2004c), Australia (Whitelaw-Weckert *et al.*, 2007), Chile (Auger *et al.*, 2007) Spain (Alaniz *et al.*, 2009), Lebanon (Choueiri *et al.*, 2009) and most recently in Canada (Petit *et al.*, 2011).

1.5 Symptoms

Scheck *et al.* (1998) described the early field symptoms of black foot disease and young grapevine decline as being virtually indistinguishable, and therefore refers to them collectively as 'decline symptoms'. In both diseases, growth of the infected grapevine slows and typical symptoms include reduced width of the trunk, shortened internodes, reduced and chlorotic foliage, and reduced leaf size (Figure 1.2a). With Petri vine decline, the longitudinal sections of trunks of declining grapevines exhibit dark brown to black streaking in the vascular tissue which turns black as the xylem becomes occluded with fungal tissue, gums and tyloses; in cross-section the symptoms appear as dots which ooze the brown or black gums forming 'pin head' extrusions (Scheck *et al.*, 1998). With black foot, the pathogen destroys the roots of grapevines and the butt of the grapevine rootstock, where it quickly

destroys vascular and cortical tissues (Gubler *et al.*, 2004). When young vines are attacked they die quickly, but as they age the infection results in a more gradual decline, and death can take more than a year to occur (Larignon, 1999). These vines have reduced water uptake and so may also dry out during summer, causing chlorosis and death of shoots. Affected vines often die during the winter, which becomes apparent with the absence of spring sprouting (Figure 1.2b) or they may sprout late and survive only to die in the following few years (Larignon, 1999).

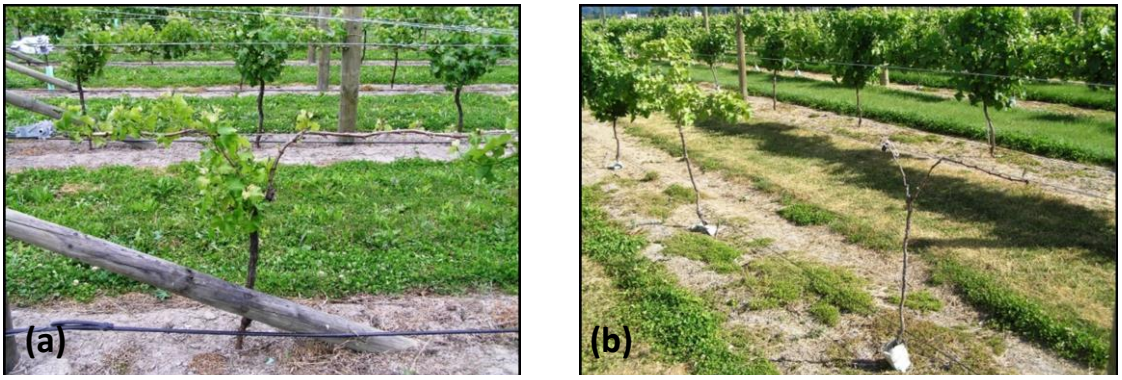


Figure 1.2 Stunted growth of an infected vine (a) and absence of growth and vine death (b).

When declining vines with black foot disease are uprooted and examined, they often show a reduction in total root biomass, low numbers of feeder roots and sunken, necrotic root lesions. Root crowns may become necrotic (Figure 1.3a) and affected vines may develop a second root system close to the soil surface to compensate for the rotted roots. A longitudinal section of the trunk shows dark purplish-black streaks in the vascular tissue that spread across the cortex and pith (Figures 1.3b and 1.3c)(Halleen *et al.*, 2004a).

Figure 1.3 Internal symptoms include brown-black discolouration of xylem tissue from the bark to the pith (a) brown-black discolouration in roots originating from the base of the rootstock (b), and necrosis in the buried sections of roots (c).

Sweetingham (1983) reported that the xylem vessels in discoloured wood were blocked with thick-walled tyloses and brown gum, which also occurred in phloem vessels. In addition, canes of affected plants showed uneven wood maturity which was often associated with the rapid desiccation events symptomatic of black foot disease (Scheck *et al.*, 1998).

1.6 Taxonomy*

Classification and identification of *Cylindrocarpon* spp. was based until recently upon morphological characteristics, although it was acknowledged that each 'species' within the genus showed great variation in morphology (Brayford, 1993). However, taxonomic and molecular investigations of the black foot isolates found in grapevines, have shown that the three most prevalent species were (1) *C. destructans* (Zinnsm.) Scholten, anamorph of *Neonectria radicicola* (Gerlach & L. Nilsson) Mantiri & Samuels, (2) *C. macrodidymum* Schroers, Halleen & Crous, anamorph of *Neonectria macrodidyma* Halleen, Schroers & Crous, and (3), *C. liriodendri* J.D. MacDon & E.E. Butler, anamorph of *Neonectria liriodendra* Halleen, Rego & Crous (Seifert *et al*., 2003; Halleen *et al*., 2004a; Petit and Gubler*,* 2005; Halleen *et al*., 2006; Alaniz *et al.,* 2007). A new genus found in South Africa *Campylocarpon* with two species *Campyl. fasciculare,* Schroers, Halleen & Crous and *Campyl. pseudofasciculare*, Halleen, Schroers & Crous both (Halleen *et al.*, 2004b) was also reported to cause black foot disease symptoms and is similar to *Cylindrocarpon* but lacking microconidia.

*Recent changes to the taxonomy of *C. destructans* and *C. macrodidymum* are briefly described in Section 1.22.

1.7 Cylindrocarpon species that cause black foot in New Zealand

When samples of declining vines were collected from the major grape growing regions of New Zealand, to determine the prevalence of black foot disease in vineyards and nurseries, this resulted in the isolation of 204 *Cylindrocarpon*-like isolates from 141 vines, which were contributed by 49 grape growers. The black foot fungi were isolated from 121 of the vines, 86% of those collected (Bleach *et al.*, 2007b). These isolates were presumptively identified by their morphology and the single-spore colonies of 204 representative isolates were sent to be identified by Mostert *et al*. (2006). From these, 60 were chosen and they used DNA

phylogenetic analysis of the internal transcribed spacers 1 and 2 (ITS1 and ITS2), the 5.8S ribosomal RNA gene and the partial β-tubulin genes to identify them as *C. destructans* (35.6%)*, C. liriodendri* (27.1%), and *C. macrodidymum* (30.5%) and at least one novel species (6.8%). In addition, Mostert *et al*. (2006) also included *Cylindrocarpon* spp. isolates from other hosts in their study. They found that the four isolates from the roots of declining apple trees reported to have apple replant disease (W. Kandula, pers comm. 2006) and that the two isolates from the rotting roots of ginseng were *C. destructans* (L. Mostert, pers comm. 2006).

1.8 Disease cycle of black foot disease

The disease cycle of these pathogens on grapevines has not been specifically studied by one group, although some research groups and individuals have investigated different aspects of the disease cycle. The behaviour of *Cylindrocarpon* spp. on other hosts has also been studied in more detail (Booth, 1966; Brayford, 1993), which has provided information about the likely disease cycle of this genus in grapevines. *Cylindrocarpon* spp. readily produce conidia and chlamydospores in culture, which indicates that these propagules are likely to be produced on the diseased roots and stem bases of infected vines. The conidia are likely to be dispersed in soil water and the chlamydospores can allow the organism to survive in the soil for a number of years (Petit *et al.*, 2011). Previous research reports (Rego *et al.*, 2001a; Halleen *et al.*, 2003; Probst, 2011) have shown that contact between these spores and the grapevine roots or callused stem bases results in high rates of infection. Infection can occur through the small wounds made when roots on the callused cuttings break off during the planting process or through the incomplete callusing of the basal ends of the cuttings which expose pith tissues (Halleen *et al.*, 2003). After infection, the pathogen invades vascular tissues, eventually restricting the uptake and subsequent transport of nutrients to the shoots and leaves and the photosynthates to the roots (Petit *et al.*, 2011). Halleen et al., (2003) and Fourie and Halleen (2001) indicated that plants can become infected in the nursery and in the vineyard but were unclear about which is the more important route. This has resulted in a number of hypotheses on the sources of primary inoculum and method of secondary spread.

1.9 Nursery sources of inoculum

The prevalence of black foot in rootstock sections of the grafted vines, mostly after being lifted from the nursery field after rooting, led to the suggestion that they were being infected in the nursery. In Spain, *Cylindrocarpon* spp. were found in 23.8% of young plants inspected (Armengol *et al.*, 2001). In Portugal, severe outbreaks were observed in young vineyards of certain grape cultivars which were linked to the same nursery or the same regional source (Rego *et al.*, 2000). When these authors investigated the causes they found that 37% of the plants were infected with *Cylindrocarpon* spp. They hypothesized that the frequency of trunk base infection in young vines indicated the potential for infection of rootstock canes prior to grafting. To determine whether infection had taken place in the rootstock mothervine blocks, canes were collected from symptomatic rootstock mothervines in Portugal and subjected to isolations. However, results showed that of the 2940 isolations made the '*C. destructans*' pathogen was absent from the majority of canes examined. The authors concluded that infection was not systemic but that it might occur through wounds made during grafting and propagation processes or in nurseries during the rooting period from soil-borne inoculum (Rego *et al.*, 2001b).

In South Africa, a survey of cuttings and nursery vines showed that *Cylindrocarpon* spp. rarely occurred in the cuttings but that the roots and rootstocks of the young vines were infected when uprooted from the nursery soil (Halleen and Crous, 2001). The subsequent studies on grafted grapevines planted in outdoor nurseries (Fourie and Halleen, 2002b; Halleen *et al.*, 2003) found that infection occurred when planted in the nursery soils. *Cylindrocarpon* spp. were isolated from less than 1% of the callused plants examined, whereas 8 months after planting, more than 50% of the plants were infected. Similar results were reported in California (Gubler *et al.*, 2004) and Portugal (Oliveira *et al.*, 2004). Standard nursery practice has involved growing the grafted plants in the same site continuously or with a 2 year rotation system, in which cuttings were planted every second year alternated with another green crop. It would therefore seem that repeated use of the same soil has led to a build-up of soil-borne *Cylindrocarpon* species which infect nursery plants (Halleen *et al.*, 2003). Primary inoculum is present in the soil, however attempts to isolate these fungi from soil where grapes are grown have not been reported to date. The above research has suggested that the reduced survival of young vines within the first year after vineyard

establishment is most likely through new infections arising from the soil inoculum in the nurseries.

1.10 Vineyard infection spread and survival

Gubler *et al.* (2004) stated that although black foot disease is linked to nursery production, since they found up to 5% of vines from some nurseries were infected with this pathogen, they believed the presence of *Cylindrocarpon* spp. in vineyard soils to be the most common primary inoculum source. This suggestion was also advanced by Oliveira *et al.* (2004), who isolated *Cylindrocarpon* spp. from 87.5% of vines with necrotic rootstock wood tissue that came from a young vineyard in Portugal. It is therefore likely that inoculum of *Cylindrocarpon* spp. can also build up in vineyard sites replanted from grapes or other fruit crops.

Newly established vineyards may be sited in soils that are already infested with species of *Cylindrocarpon,* since they are common soil inhabitants, occurring as saprophytes or weak pathogens and often associated with roots of herbaceous woody hosts (Brayford, 1993). The *Cylindrocarpon* species used in the current research were reported as pathogenic (Probst 2011). Pathogenic strains of *C. destructans* were reported to cause plant losses during propagation of conifer and fruit tree nursery stocks, and to be frequently associated with or the cause of seedling blights, basal rots of bulbs and root rots of a diverse range of plants (Samuels and Brayford, 1990). *Cylindrocarpon destructans* has a wide geographic distribution, in both parasitic and saprophytic forms (Seifert *et al.*, 2003). *Cylindrocarpon* isolates from roots of declining apple trees in New Zealand were identified as *C. destructans* and found to be similar to isolates from New Zealand grapevines (Mostert *et al.*, 2006). This suggested that if declining apple orchards were converted to vineyards, they could provide an inoculum source for development of black foot, and Bonfiglioli (2005) proposed that the high rate of black foot infection in New Zealand vineyards could be due to the number of vineyard sites previously planted with apple trees.

Cylindrocarpon destructans is of particular concern to the ginseng industry where it causes root rot and can destroy this crop. In ginseng it is proposed that secondary spread might occur via aerial dissemination of *C. destructans* conidia produced from the surface of rotted roots (Howard *et al.*, 1994). In grapes which are deep-rooted, the conidia are more likely to

be produced within the soil and may be spread by splash onto wound sites made during pruning and removal of trunk shoots (called 'bud-rubbing'). Probst (2011) reported that infection occurred through cane and trunk wounds and progressed downward to the bases of the vines. However, since the most commonly reported infection sites are the underground portions of grapevine trunks (Fourie and Halleen 2001; Halleen *et al*., 2004b; Gubler *et al*., 2004), it is more likely that spread of the pathogens in the vineyard occurs through movement of infested soil. The pathogen may also spread through direct contact of roots between neighbouring vines or in wetter areas via water movement through or over the soil.

1.11 Environmental and vineyard factors

As is the case with *Cylindrocarpon* species that cause disease on other crops, environmental factors and host stress play an important part in disease development (Brayford, 1993). *Cylindrocarpon* spp. may exist as harmless organisms living within the plant tissue (endophytes) until plant stress conditions favour development of the disease (Oliveira *et al.*, 2004). In grapevines, stress may be caused by other fungal infections. Indeed, Rego *et al*. (2001) noted that other wood fungi, such as *Fusicoccum* Corda*, Phomopsis* Sacc. & Roum*, Truncatella* Steyaert and *Pestalotiopsis* Steyaert, species colonized the canes of rootstock mothervines which possibly weakened cuttings, predisposing them to the subsequent infection by *Cylindrocarpon* spp. Other factors that may contribute to plant stress and make young grapevines susceptible to infection include malnutrition, poor water drainage, heavy cropping loads on young plants and poor planting techniques (Brayford, 1993; Fourie and Halleen, 2001; Halleen *et al.*, 2004b). Severe losses have been reported from grapevines planted and grown on susceptible rootstocks in heavy, poorly drained soil (Gubler *et al.*, 2004).

Observations of vineyards in California have indicated that 'J' rooting and water stress may be partially responsible for vine decline symptoms (Gubler *et al.*, 2004). 'J' rooting, which was also observed in New Zealand vineyards during these studies, is a condition where the root system of the newly planted vine points upward, often as a result of poor planting technique as well as compact or poorly prepared soil. This condition limits the ability of the root to take up water, minerals and nutrients (Gubler *et al.*, 2004). Root system expansion is partly dependent on environment and genetic factors, but soil penetration can be increased

by breaking up hardpans with soil ripping. Management of this disease is improved by selecting healthy plants that have the rootstock variety most suitable for the environment and by careful planting in well prepared, well-drained soil (Gubler *et al.*, 2004).

1.12 Management of Cylindrocarpon black foot disease

Few specific recommendations for the control of back foot disease are currently available. Fourie and Halleen (2002a) recommended that soil treatment and preparation to reduce soil pathogen populations could be further investigated as a means of managing black foot disease and reducing possible infection and spread. Later, Halleen *et al*. (2006a) recommended some management strategies that focused on nursery and vineyard production systems, which included the prevention and/or correction of predisposing stress factors.

1.13 Rootstock varieties

The most effective method known to control phylloxera was the use of grapevines grafted onto phylloxera-resistant rootstocks (Powell and Herbert, 2005). However, other disease problems have arisen since they became widely used. Scheck *et al.* (1998) reported that in California, grapevine death caused by root rot had increased since the introduction of phylloxera resistant rootstocks and that vine decline was prevalent in rootstocks planted for phylloxera control. The anecdotally reported susceptibility of grapevines to *Cylindrocarpon* spp. has varied between cultivars and countries, however there has been little clear information on cultivar susceptibility (Halleen *et al.*, 2006; Rego *et al.*, 2000; Scheck *et al.*, 1998). Evaluation of the susceptibility of the most commonly used grapevine rootstocks in Spain found that all rootstocks inoculated with *C. liriodendri* and *C. macrodidymum* became infected by the disease (Alaniz *et al.*, 2010). In California, Gubler *et al*. (2004) found that no rootstocks were completely resistant to this pathogen, but *Vitis riparia* (O39-16) and Freedom had moderate resistance to *C. destructans.*

In New Zealand, experiments to test the susceptibility of rootstocks to black foot disease were undertaken in an attempt to find resistance among the commonly grown rootstock varieties. In collaboration with Lincoln University, Ian Harvey of PLANTwise™ carried out greenhouse experiments to test the resistance of the 14 and 11 rootstock varieties, available

in 2005 and 2006, respectively. Results showed that no rootstocks were completely resistant to infection, however the varieties *Riparia gloire* and R140 were most tolerant to black foot infection, with Rupestris St George, 5C and 3309 having some tolerance to infection (Harvey and Jaspers, 2006).

Bleach *et al*. (2007a) (Appendix A.4, data not provided) tested the susceptibility of six rootstock varieties to *Cylindrocarpon* spp. infection under field conditions and found that no rootstock was completely resistant to infection but some rootstocks were tolerant to a degree, and that there was a difference in susceptibility to black foot disease depending on the amount of inoculum in the soil. In soils with heavy disease pressure rootstocks 5C and 3309 were the most resistant to black foot infection compared to Schwarzmann, 420A, *Riparia gloire* and 101-14 which were the least resistant to infection, respectively (unpublished data). No rootstock variety was completely resistant to the black foot pathogens, a conclusion which agreed with Gubler et al. (2004). In soil with low disease pressure, rootstocks Schwarzmann and *Riparia gloire* were the most resistant to black foot infection and 420A the most susceptible to the pathogen (unpublished data). The results of this rootstock experiment were presented as an oral presentation to New Zealand Winegrowers at the 13th Annual Romeo Bragato Conference in Auckland, New Zealand. In both this experiment and that conducted by Harvey and Jaspers (2006) it was observed during the assessment of the symptomatic vines that the pathogen frequently initiated infection through the roots and trunk bases of the rootstock (I C Harvey, pers comm. 2007) indicating this to be an important aspect to investigate when considering disease management.

1.14 Biological control with Trichoderma products

Trichoderma spp. can indirectly control phytopathogens by competing for space and nutrients, or through the secretion of antibiotic volatiles and/or diffusible metabolites, which modify soil conditions promoting growth and plant defence mechanisms (Howell, 2003). There is a great body of evidence to support the efficacy of *Trichoderma* strains as biocontrol agents against different phytopathogenic fungi (Benítez *et al.*, 2004). In addition, they have been reported to have a stimulatory effect on plant growth (Hohmann *et al.*, 2011; Naseby *et al.*, 2000). *Trichoderma* spp. are reported to be especially active against soil borne microorganisms (Howell, 2003) such as *Rhizoctonia solani* Kühn, which causes crown

and root rot in tomatoes (Benítez *et al.*, 2004) and *Sclerotium cepivorum*, Berk. the causal agent of onion white rot (Kay and Stewart, 1994).

Early research into the control of black foot disease by Fourie *et al*. (2001) investigated the benefit of using *Trichoderma* products. They dipped grapevine rootstock material for 5 s into a *Trichoderma* suspension (Trichoflow-T™ Agrimm Technologies Ltd., Christchurch, New Zealand) before and after grafting. After callusing, the vines were planted in soil that had previously been treated with Trichopel-R™ (Agrimm Technologies Ltd., Christchurch, New Zealand) and after planting the root zone was drenched with Trichogrow™ (Agrimm Technologies Ltd, Christchurch, New Zealand) on a monthly basis with a total of six applications. Results showed that overall the *Trichoderma* treatments increased root development compared to the untreated control plants (mean root dry weights of 36.46 and 25.73 g, respectively) and reduced the incidence of *Cylindrocarpon* spp. in the roots of the *Trichoderma* treated plants compared to the control plants (1.6 and 2.8%, respectively) (Fourie *et al.*, 2001).

Field trials were conducted at two nurseries in the 2003 season and repeated in the 2004 season, by Halleen *et al*. (2007) who tested the efficacy of *Trichoderma* treatments in preventing natural infection of the roots and basal ends of grafted nursery grapevines from black foot pathogens in naturally infested soil. Basal ends of the rootstocks were dipped for 1 min in various treatments which included Trichoflow-T™. In another treatment, Trichopel-R™ was added to the planting furrows and after planting, the root zones were drenched with Trichogrow™ at monthly intervals with a total of 6 applications. *Trichoderma* spp. were not isolated from the roots of plants in either season, nor were the incidences of *Cylindrocarpon* spp. less than in the water control ($P>0.05$) in either the 2003 or the 2004 season. A possible explanation for the low re-isolation percentage of *Trichoderma* spp. observed in the harvested plants was thought to have been due to insufficient systemic colonisation of the basal ends of rootstocks due to the short dip treatment of 1 min (Halleen *et al.*, 2007).

1.15 Mycorrhizae

Arbuscular mycorrhizal fungi form associations with plants that improve their nutrient uptake and stress tolerance and improve the resistance of plant root systems to soil borne diseases (Perrin, 1990). However, the most effective interaction between plant species and

mycorrhizal fungi is with the selection of an arbuscular mycorrhizal species that is specific to the host plant species (Plenchette *et al.*, 1983). In grapevines, greater incidence of vesicular arbuscular mycorrhizal colonisation was shown to decrease the ability of pathogens to find infection points (Joubert and Archer, 2000) and increased the survival of plants under non favourable growing conditions and in marginal soils (Lioi and Giovannetti, 1987).

The effect of a mycorrhizal association in reducing incidence of black foot caused by *C. macrodidymum* was investigated under controlled conditions using *Vitis rupestris* cv. St. George colonised by the mycorrhizal fungus *Glomus intraradices* (INVAM CA 501) (Petit and Gubler, 2006). The callused cuttings were planted into soil which had mycorrhizal inoculum added to allow colonisation of the roots before the plants were challenged with the *Cylindrocarpon* inoculum. After harvest, mycorrhizal colonisation was found to be high in roots of the mycorrhizae treated plants, being 48.3% for non-infected control and 54.5% for those infected with *C. macrodidymum.* At assessment, mycorrhizal plants had significantly fewer symptoms than non-mycorrhizal plants, with 37% less necrotic leaf area and 40% fewer lesions on diseased roots. Plants that were inoculated with the pathogen but had no mycorrhizae, had 50% lower mean shoot dry weights and 30% lower mean root dry weights than the non-pathogen inoculated plants (Petit and Gubler, 2006). The findings of this study showed that even though *C. macrodidymum* was found in both mycorrhizae and non-mycorrhizae inoculated plants, disease severity was significantly less in the roots of mycorrhizae inoculated plants.

Young vines with arbuscular mycorrhizae are better able to resist transplantation shock (Linderman and Davis, 2001), which occurs when the roots of young vines are damaged during planting (Waschkies *et al.*, 1993). Such damage can restrict the plants' ability to uptake water and nutrients (Linderman and Davis, 2001). Waschkies *et al*. (1993) suggested that mycorrhizae colonisation alleviated transplantation shock in young grapevines, by improving water relations and nutrient access when the root systems of young vines were damaged or compromised during the planting process. Indeed, van Rooyen *et al.* (2002) reported that one year old mycorrhizal inoculated grapevines cultivated in a controlled atmosphere environment, had improved photosynthetic rates which they related to "improved water relations". They concluded that the improved photosynthetic performance of the grapevine during the transplantation period "potentially" improved survival of the

grapevine during the initial growth stages (van Rooyen *et al.*, 2002). In New Zealand field experiments, colonisation by arbuscular mycorrhizae species *G. intraradices* and *G. mosseae* of roots of grapevine cuttings planted into soil infested with *Cylindrocarpon* spp. was thought to have contributed to improved plant growth since root dry weights were significantly increased at the Auckland site (114%) and increased by 22% at the Blenheim site compared to control plants, however *Cylindrocarpon* infection levels were not reduced (Bleach *et al.*, 2008) (Appendix A.3). Regardless, this experiment demonstrated that grapevine cuttings colonised with mycorrhizae that were grown in *Cylindrocarpon* infested soil had improved plant vigour.

1.16 Fungicides

The application of chemicals to control fungal trunk pathogens in nurseries and vineyards is unlikely to be totally effective as chemical sprays and dips do not penetrate dormant grapevine cuttings sufficiently to control the organisms that inhabit the phloem and xylem tissue (Groenewald *et al.*, 2000; Jaspers, 2001; Waite and May, 2005). However, they may be used to protect the potential infection courts.

In Portugal, Rego *et al.* (2006) carried out *in vitro* fungicide screening and found that prochloraz, benomyl, carbendazim + flusilazole and cyprodinil + fludioxonil inhibited mycelium growth of '*C. destructans*' (which was later identified as *C. liriodendri*), whilst tebuconazole and difenoconazole were less effective. Among the fungicides that effectively reduced mycelium growth of the pathogen, only the mixture cyprodinil + fludioxonil inhibited conidium germination. The strobilurin fungicides, azoxystrobin and trifloxystrobin and the phenylsulfamide tolylfluanid, while less effective in inhibiting mycelium growth, inhibited conidium germination of the *C. liriodendri* pathogen (Rego *et al.*, 2006). The authors also tested fungicides selected from their *in vitro* experiments under greenhouse conditions. They reported that after a three months' growth, the growth parameters and disease incidence assessed from the uprooted plants showed that benomyl, tebuconazole, carbendazim + flusilazole and cyprodinil + fludioxonil "significantly improved plant growth and decreased disease incidence" compared to control plants. Nascimento *et al*. (2007) also reported that fungicide dips of cyprodinil + fludioxonil, carbendazim + flusilazole and tebuconazole and a foliar spray of chitosan were effective in a greenhouse experiment using potted grapevines as the incidence of *C. liriodendri* was reduced. In that experiment the

treated cuttings were transplanted into a potted substrate which had been artificially infested with a *C. liriodendri* isolate, after which the chitosan treated plants were sprayed. After 3 months growth all treated plants had reduced incidence of *C. liriodendri* compared to the controls but this was only significant for the plants that were treated with cyprodinil + fludioxonil and chitosan.

Soaking propagation material in fungicides during different stages of grafting has resulted in a reduction of *Pa. chlamydospora* and *Phaeoacremonium* infection in the basal end and graft union of nursery plants (Fourie and Halleen, 2004; Fourie and Halleen, 2006; Gramaje *et al.*, 2009). Indeed Fourie and Halleen (2006) showed that soaking propagation material prior to grafting in chemical and biological treatments: benomyl, Bio-sterilizer, Bronocide®, Chinosol®, captan, didecyldimethylammonium chloride, (Sporekill™) or *T. harzianum* (Trichoflow-T™) had reduced *Pa.chlamydospora* and *Phaeoacremonium* infection in the basal end and graft union of the grapevines (Fourie and Halleen, 2006). However when benomyl, captan and Sporekill™ were tested to reduce the incidence of the black foot pathogens *Cylindrocarpon* and *Campylocarpon* spp. there was no significant treatment effect. The authors suggested that this was most likely due to the very low incidence of these species in the basal ends of the rootstocks in their experiments. These pre-soaking treatments have been reported to have little effect on the black foot pathogens (Halleen *et al.*, 2003), possibly because infection by black foot pathogens is believed to originate from infested nursery soils and not during propagation practices (Fourie and Halleen, 2006).

Further testing by Halleen *et al*. (2007) using the four most effective fungicides from their *in vitro* experiments were evaluated for their effect under field conditions, in naturally infested soil at two different nursery sites, over two seasons. After 7 months' growth, Halleen *et al*. (2007) found that the incidences of the black foot pathogens in the rooted cuttings were "not significantly and/or consistently reduced nor plant growth improved" in either season by the majority of chemical or biological treatments. In both seasons, benomyl + Nu-Film 17 was relatively effective but the most effective treatment, with significantly lower incidence of black foot pathogens than the control plants (16·8%), was the hot water treatment (0%) (Halleen *et al.*, 2007).

Phosphoric acid salts are thought to protect plants from root pathogens by "stimulating host resistance" (Schwinn and Staub, 1995) to pathogens such as *Phytophthora* and *Pythium* spp.

(Marais and Hattingh, 1986). The positive effect of phosphoric acid applied as foliar sprays in a glasshouse experiment was reported by Ferreira (1998). In his glasshouse experiment, phosphorus acid (Phytex) had an inhibiting effect on *Pa. chlamydospora* and so the incidence of Petri disease lesions in artificially inoculated vines. Di Marco *et al*. (2000) reported that another product Fosetyl-Al, which is rapidly degraded to phosphorous acid, showed promising results in the management of esca disease of grapevines caused by Petri disease fungi and *Fomitiporia punctata* (Fr.) Murrill. In contrast, Fourie and Halleen (2004) reported indifferent effects of Phos-guard 400SL, (Ocean Agriculture, South Africa) used as a 60 min pre-plant drench of rootstock cuttings prior to grafting. After 8 months growth in a greenhouse and in two naturally infected commercial field nurseries, plants that had been treated with the phosphoric acid product were found to have disease incidence "similar to or higher than" that of the water treated control (Fourie and Halleen, 2004). Conversely, Rego *et al*. (2006) reported that plants that had been dipped for 50 min in a Fosetyl-Al suspension and then grown for 3 months in substrate infested with *C. destructans* (which was later identified as *C. liriodendri*), had significantly reduced disease incidence compared to untreated control plants, although this product had not shown *in vitro* efficacy (Rego *et al.*, 2006). The shorter growth period of 3 months, compared to an 8 months growth period as reported by Fourie and Halleen (2004) may have influenced this result.

There are no fungicides registered in New Zealand for the control of black foot disease although some products have demonstrated efficacy in overseas studies and are currently used in New Zealand to protect grapes against foliar and fruit pathogens like Botrytis bunch rot and among these are: captan, carbendazim, cyprodinil and fludioxonil. However, these products have not been tested against black foot disease in New Zealand under New Zealand conditions.

1.17 Hot Water Treatment

Research in South Africa (Crous *et al.*, 2001; Fourie and Halleen, 2004), Australia (Edwards *et al.*, 2004; Waite and May, 2005; Waite and Morton, 2007) and California (Rooney and Gubler, 2001) has examined the efficacy of hot water treatment (HWT) to sanitise propagation material. There are two regimes currently used on grapevines: a short duration of 5 min at 52-55°C for the control of external pests and pathogens on dormant grapevine cuttings and rootlings and a longer HWT of 50°C for 30 min for the control of both

exogenous and endogenous pests and pathogens. Crous *et al*. (2001) tested the effectiveness of HWT on the endophyte population of grapevines, which included *Botryosphaeria* spp., *Cylindrocarpon* spp., *Fusarium* spp. and *Phomopsis* spp. Cuttings were immersed in a hot water bath at 50°C for 30 min, immediately followed by a cold water bath for 30 min. Isolations made immediately after the treatment found no living fungal pathogens. However, the number of living fungal colonies isolated from the HWT cuttings, 6 months after planting in a field nursery, were not significantly different from the untreated cuttings. This clearly shows that the advantage of plants being free of endophytic fungal pathogens may be short lived (Crous *et al.*, 2001).

HWT has long been used as a prophylactic treatment to rid young grafted vines of some pathogens and pests but it can cause detrimental effects to the viability of treated plant materials (Ciancio *et al.*, 2008). Until now, the standard HWT protocol has been 50°C for 30 min but some researchers have suggested that it may not always be effective while others have reported that it may result in unacceptable damage to young vines (Rooney and Gubler, 2001; Whiting *et al.*, 2001). Critical factors to consider for ensuring efficacy without harm are water temperature and treatment duration, the volume of plants to be treated with respect to the amount of water used, as well as the physiological state of the dormant plant material, and pre-treatment hydration times (Fourie and Halleen, 2004; Waite and May, 2005). Waite and May (2005) reported the effects of different hydration times and HWT protocols for dormant cuttings and concluded that the standard treatment (50°C for 30 min) could be used successfully in a commercial situation, but may occasionally result in unexplained failure of young rooted HWT vines in the vineyard.

Waite and Morton (2007) and Graham (2007a) suggested that the tolerance of plants, and their accompanying pathogens to HWT might be affected by the climate in which cuttings or plants were previously grown. In New Zealand, Graham (2007a) found that grapevine cuttings that were HWT at 47°C for 30 min, reduced “known pathogens and endophytes” in grafted rootstock to 3% compared to the untreated controls (15%). Vine mortality was also reduced to less than 10% in vines that were treated at a lower temperature (45-47°C for 30 min) compared to vines that were HWT using the industry standard (60%) (Graham, 2007a). A reduced HWT temperature could lessen the risk of any negative effects caused on the propagation and planting material. Since Halleen *et al.* (2007) showed that this treatment

was highly effective, HWT of dormant nursery plants should be considered in an integrated strategy for the proactive management of black foot disease. However, it is possible that the putative, negative effects of this treatment could be reduced by developing a protocol more suitable to the cool climate viticultural regions of New Zealand, which requires further study.

1.18 Chemical soil fumigants

Fumigation with methyl bromide has been used for decades as a standard treatment for eliminating soil-borne disease and pests in replant systems. However, to comply with the Montreal Protocol on ozone-depleting chemicals, use of methyl bromide for soil fumigation was banned in New Zealand and other developed countries from 2008 (Horner *et al.*, 2007). This has led to a search for alternative strategies to manage soil-borne diseases, but so far none of the chemical alternatives identified have the full spectrum of activity and versatility of methyl bromide as a pre-plant soil fumigant (Martin, 2003). Chemical alternatives to methyl bromide are subject to continuing review and regulation, therefore the prospects for long term registration of any new fumigants currently under development are unknown (Duniway, 2002). Non-chemical alternatives were not adequately developed or effective enough to meet current needs, and so growers have used the known chemical alternatives to methyl bromide for fumigation of soil. In the United States, chloropicrin (trichloronitromethane, NiklorChemical Co., Long Beach, Calif.), 1,3-dichloropropene (1,3-D, Telone®, Dow AgroSciences, Redeck, N.C.), and metam sodium (sodium *N*-methyl dithiocarbamate, BASF, Research Triangle, N.C.), are the only soil fumigants currently registered and available that have enough "broad-spectrum" activity to be considered as replacements for methyl bromide (Duniway, 2002). In Australia the use of chloropicrin has increased as methyl bromide use has declined (Porter *et al.*, 1999). Although chloropicrin and 1,3-dichloropropene are able to provide significant control of many plant pathogens in soil, and growth stimulation in annual crops, they only provide limited control of weeds or other residual plant materials in "soils of concern" in nursery production systems (Duniway, 2002).

Cylindrocarpon destructans has been associated with crown and root disease of strawberries in New Zealand (Bonfiglioli, 2005) and overseas (Fang *et al.*, 2011; Mattner *et al.*, 2008). Fumigation of strawberry beds with methyl bromide and rotation with grain crops has been used to control root diseases in this crop (Fang *et al.*, 2011). The potential for alternative

fumigants to replace methyl bromide was evaluated in several high elevation strawberry nurseries in California (Kabir *et al.*, 2005). The treatments evaluated for control included methyl bromide plus chloropicrin (MBPic), iodomethane plus chloropicrin (IMPic), methyl iodide (iodomethane) plus chloropicrin (Impact), 1, 3-dichloropropene plus chloropicrin (Telone®C35) followed by dazomet, chloropicrin (Pic) followed by dazomet and a non-fumigated control. The fumigants were applied to the soil and covered with polyethylene for 7-14 days. Several months after planting the strawberry plants, runner plant production and marketable fruit yield were assessed. The authors reported that in the high elevation nurseries the use of non-methyl bromide pre-plant fumigants produced runner plants of "sufficient quality and vigour" to be commercially acceptable in strawberry plant nurseries and so the non-methyl bromide fumigants IMPic, Impact or Telone C35 followed by dazomet, and Pic followed by dazomet were potential alternatives to methyl bromide fumigation in the industry (Kabir *et al.*, 2005). The pathogen colonisation of the planting material was also assessed but the results were not reported.

In New Zealand trials conducted from 1998 to 2006, in commercial strawberry gardens and in the Roselea Research Garden, Hawkes Bay the 'pathogen killing ability' of alternative soil fumigants were tested. Results showed that 1,3-dichloropropene + chloropicrin (Telone®C35) and chloropicrin performed well, with fruit production equal to or only slightly lower than when the soil was treated with methyl bromide (Horner *et al.*, 2007). However, the authors reported that in other experiments fumigant movement through the soil, pathogen kill and plant health had generally been inferior to that achieved with methyl bromide. In soil which was excessively wet during preparation and fumigation, Telone®C35 and chloropicrin were inferior to methyl bromide, producing relatively poor crops. In such conditions, methyl iodide was the only fumigant for which plant performance was close to that achieved with methyl bromide. Although methyl iodide appeared closest to methyl bromide in performance, testing of this product has been very limited, and it has not yet been accepted as an alternative to methyl bromide in commercial gardens (Horner *et al.*, 2007). Products such as dazomet (Basamid®, BASF New Zealand Ltd.,) and metam sodium (Fumasol™, Elliot Technologies Ltd. New Zealand) may also be useful alternatives, however these products are only effective to the depth of incorporation as they do not move well through soil and their performance is poor in wet conditions (Horner *et al.*, 2007). Further studies are required to evaluate the efficacy of these products, as well as cost-benefit analyses, and the feasibility of

integrating them into grape production systems. In field experiments in sites at Auckland and Blenheim, soil fumigants Telone C35 and Chloropicrin (Leicesters Soil Fumigation, Napier, New Zealand) reduced *Cylindrocarpon* incidence in grapevine cuttings grown in *Cylindrocarpon* infested soil by 16 and 17% and 24 and 42%, respectively, compared to untreated controls (Bleach *et al.*, 2008; Appendix A.3). These marginal results suggest that soil fumigation may not be a viable control for black foot disease since the chemicals are expensive, can only be applied by a certified contractor (B. Leicesters, pers comm. 2007) and are likely to eliminate beneficial soil fungi.

1.19 Soil bio-diversity

The addition of compost to soil improves both soil quality by improving the chemical and physical properties of the soil and soil health by enhancing 'ecological characteristics' (Karlen *et al.*, 1997). The improved soil supports the development and maintenance of microbial communities (Hoitink and Fahy, 1986) which include taxonomic groups such as bacteria, fungi and other soil organisms (van Bruggen and Semenov, 2000) and these organisms are thought to suppress root diseases (Hoitink and Fahy, 1986; Hoitink and Changa, 2004). Many soil organisms compete with and can kill or suppress, plant pathogenic fungi (Sturtz *et al.*, 1997) by mechanisms which include "competition, antibiosis, hyper-parasitism and the induction of systemic acquired resistance" (SAR) in the host plant (Hoitink *et al.*, 1997).

Systemic acquired resistance allows plants to resist attack by insects and pathogenic invaders and also recover from disease (Sticher *et al.*, 1997). Pathogen attack triggers a salicylate burst in the plants (Vasyukova and Ozeretskovskaya, 2007) which activates the systemic acquired resistance pathway. Vasyukova and Ozeretskovskaya (2007) reported that: "the resistance depends on the ability of the salicylate compound to inhibit the enzymes of the antioxidant system of plants, which results in the accumulation of oxygen species and the expression of defence genes". It is sometimes called 'broad spectrum' because this resistance is induced by a wide range of pathogens, not only those that cause tissue necrosis (Sticher *et al.*, 1997).

Fungistatic activity associated with significant populations of soil bacteria is also expressed in soil with increased organic matter content and higher microbial activity (Sturtz *et al.*, 1997). *In vitro*, bacteria isolated from organically managed vineyard soils were shown to be

antagonistic towards *C. destructans* and *F. oxysporum* (Sturtz *et al.*, 1997), which were hypothesized by Whitelaw-Weckert (2004) to possibly occur through the "production of diffusible antibiotics". However, Sturtz *et al*. (1997) considered that high levels of microorganism activity increased competition in the soil, which reduced pathogen populations and potentially resulted in disease suppression. Gugino and Travis (2003) reported that adding of compost and antagonistic microorganisms to soil reduced the incidence of *C. destructans* populations in soils. They used serial dilution plating to monitor *C. destructans* populations in soils amended with 0, 10, 25 and 50% compost. The increased concentration of the compost resulted in a reduction in the *C. destructans* population. They also found that several other microorganisms isolated from the composts demonstrated antagonism towards *C. destructans* species (Gugino and Travis, 2003). The benefit of "increased soil biological activity" through the addition of organic matter to phylloxera infested vineyards was reported by Lotter *et al*. (1999). They found that after the addition of organic matter, root disease in phylloxera infested plants caused by pathogens such as *Fusarium* and *Cylindrocarpon* spp. was reduced by 70% in plants grown in the organically managed vineyard compared to a conventionally managed vineyard.

Hoitink and Changa (2004) reported that composts used in the nursery industry could effectively suppress some root pathogens as was shown during the 1950's in the nursery industries in the United States and Australia. After nurserymen began to introduce bark from several tree species as peat substitutes in container media, they observed that the 'bark amended potting mixes' seemed to naturally suppress Phytophthora and Pythium root rots (Hoitink and de Ramos, 2004). Since the microorganisms present in composted pine bark were naturally suppressive to some soil-borne plant pathogens, Hoitink and Changa (2004) concluded that inoculants could be added to soil to maximise the spectrum of natural disease control and induce systemic resistance to disease in the plants (Hoitink and Changa, 2004; Hoitink *et al.*, 1997).

As well as root disease suppression, other advantages of the use of composts as soil amendments is the use of waste products such as bio-solids, food waste and manures to provide essential major and minor nutrients to soils and container media (Hoitink and de Ramos, 2004). Recycling through composting is more commonly the preferred strategy for waste treatments; accordingly, composts are becoming available in greater quantities

(Hoitink *et al.*, 1997). A current example is the Living Earth Compost™ described as ”rich, dark, weed free compost”, produced from “100% green-waste from councils, landscapers and home collections” which is certified organic ('Bio-Gro' New Zealand™) and suitable for all plants (http://www.livingearth.co.nz).

The New Zealand Institute for Plant and Food Research Limited began field trials in 2007 to assess the qualities of a Living Earth™ compost and one other compost supplied by Transpacific Industries Limited, Timaru, New Zealand. The scientists assessed the effects on establishment and growth of forage kale and annual ryegrass as well as the associated soil quality changes under each crop and the potential of the compost to reduce mineral fertilizer requirements (Horrocks *et al.*, 2010). The research started as a three year trial at Karina Downs near Timaru, but during its third year, the trial was extended to a five year project, and included plantings at Lincoln, Bankside and Marshlands in Canterbury (G.Hemm, pers. comm. 2011). Results showed significant increases in dry matter yield of both kale and ryegrass with increasing compost, being similar for both types. In the first and second year the dry matter yield of kale, which received the most compost (110 t/ha), was 50% more than the control. The total dry matter biomass for the ryegrass, which was measured prior to grazing in February and March 2008 was 200 and 400% more, respectively, than for the control plots (Tregurtha *et al.*, 2009a; Horrocks *et al.*, 2010). Hemms, (pers. comm. 2011) also stated that “these results were in response to a single compost application applied only in the first year; accordingly the first year’s response was more impressive, but tailed off in the second year. Initial measurements made on soil properties indicated that numerous benefits from the addition of compost were expected to increase as the trial proceeded (Tregurtha *et al.*, 2009b) however they did not elaborate on what those benefits would be. The extended five year project (now in progress) with field trials and laboratory experiments is intended to establish guidelines for the sustainable use of these compost treatments.

Vermicomposting is the biotechnological process using epigeic earthworms and soil microorganisms to compost organic matter and industrial waste material to a higher valued product, called ‘vermicompost’. Epigeic earthworms, which inhabit the upper mineral soil layer, carry out mineralisation of plant surface residues by burrowing, ingesting and egesting to produce castings composed of residues plus soil. The castings (vermicompost) are reported to be rich in microbial activity, nutrients, and plant growth regulators and to have

“pest repellent attributes” (Munroe, 2007 in Medany, 2011). This egested material can then be transported to lower soil profiles by the anecic and endogeic earthworm types which burrow (Medany, 2011) and stimulate the physical, chemical and biological properties of the soil to improve soil biodiversity and health (Kizilkaya *et al.*, 2011) and increase plant production and reduce plant disease (Meghvansi *et al.*, 2011; Simsek-Ersahin, 2011).

The utilisation of vermicompost products, either as solid organic fertilisers or liquefied, for the inhibition of plant disease is still under investigation in the United States, Canada, Japan, France and New Zealand, but as a new domain vermicomposting provides a wide variety of alternatives for biodegradable organic waste management and agrochemical-free food production (Simsek-Ersahin, 2011). Wormtech™ New Zealand Ltd has produced *Eisenia feti*, (Oligochaeta, Lumbricidae) which are epigeic composting earthworms, for supply to composting facilities that dispose of organic waste. The vermicompost is formulated into solution with the addition of 10% humic acid which is believed to enhance the activity or concentration of extracellular enzymes known to bond to humic matter during vermicomposting (Benitez *et al.*, 2000). The Wormtech™ formulation is intended as a substitute for solid compost to aid the breakdown of mulched prunings and improve the decomposition of plant debris by promoting biological activity in the soil (Wormtech™, 2006). Wormtech™ New Zealand Ltd has developed application guides for its use as soil drenches and as soil and foliar sprays which are stated to be suitable for a range of horticultural and nursery crops which include grape, kiwifruit, avocados and blueberries. In field experiments in sites at Auckland and Blenheim liquid worm compost provided by Wormtech™ New Zealand Ltd, reduced *Cylindrocarpon* incidence in grapevine cuttings grown in *Cylindrocarpon* infested soil by 30% in Blenheim. However compost provided by 'Bio-Gro' New Zealand™ Ltd was not effective (Bleach *et al.*, 2008), (Appendix A.3).

1.20 Rotation crops and biofumigation

Crop rotation involves the successive planting of different crops in a site. This traditional method reduces pathogen survival because the types of break crops do not support the pathogens and the periods between the same crops are usually longer then the survival period of the host specific pathogens from that host. Crop rotation has been effective in controlling “bacterial blight” of wheat, barley, grasses and rye and “smut” of corn and “scab disease” of all these crops (Peel, 1998). Research in North Dakota over 8 years showed that

the average benefit of rotating wheat with barley improved yields (4%) compared to continuous wheat crops but that greater benefits were reported when wheat or barley were rotated with an unrelated crop such as legumes (soybean), with 14% increased yield (Peel, 1998). Crop rotation may have the greatest impact on increased yield in host plants likely to be affected by the build-up of soil-borne pathogens as can be the case in mono-cropping systems.

Crop rotation may also improve soil fertility and soil organic matter content, especially if the alternating crops include hosts such as cereals or legumes that are incorporated into the soil rather than being harvested. This has been shown to restore structural damage caused by mono-cropping (McLaren and Cameron, 1996) and in turn to support sustained production (Mitchell *et al.,* 1991, in Karlen *et al*., 1994). With reduced disease pressure, improved soil fertility and structure and more efficient soil water management, Peel (1998) also reported that yields and consequently profits were increased. Sustainable farming practices support the continued use of rotation cropping, however in more recent years some farmers have moved away from crop rotation. Due to the highly competitive nature of their industries, they have needed to focus on meeting market demands in the short term. This has led to increased use of nitrogen fertilisers, herbicides for weed control and pesticides for insect and disease control even though the sustainability of these practices may be questionable (Karlen *et al.*, 1994).

Use of the *Brassicaceae* as rotational crops can provide a further benefit because the volatile compounds released from the roots and plant residues can naturally suppress some soil-borne pathogens and pests (Brown and Morra, 1997). The volatile allelochemicals are released into the soil from the damaged plant tissue when myrosinase enzymes hydrolyse glucosinolates contained in the plant (Kirkegaard *et al.*, 1993; Mattner, 2001). Among the degradation products of glucosinolates, isothiocyanates (ITCs) have been reported as the most potent (Rosa, 1999). The efficacy of plant residues from the *Brassicaceae* for control of soil-borne pathogens and parasites, a process called 'biofumigation' (Kirkegaard *et al.*, 1993), has typically been attributed to the toxicity of these glucosinolate hydrolysis products (Brown and Morra, 1997). The optimum suppressive effects can be achieved if the brassica plants are grown to flowering and then incorporated into the soil (Rosa, 1999). The

fumigation properties and biological effects of the *Brassicaceae* could be used as a sustainable alternative to methyl bromide (Rosa, 1999).

The potential of the biofumigant crop Indian mustard (*Brassica juncea*) was evaluated in grapevine nursery fields and *in vitro*, as a possible alternative for methyl bromide and metham sodium for the control of *C. destructans* (Stephens *et al.*, 1999). *In vitro* the Indian mustard shoot macerates inhibited mycelium growth of '*C. destructans* previously isolated from stunted nursery grapevines' by almost 50% of the control, however *in vivo* the Indian mustard was associated with fewer living grapevine plants (87.5%) compared to the control (93.5%). The authors found that there was a massive increase in numbers of *Pythium* propagules immediately after incorporation which remained high for more than 78 days. Since the grapevines were planted 18 days after the initial incorporation of the mustard, the authors suggested that the increased *Pythium* propagules may have caused the plant deaths rather than a direct allelopathic effect (Stephens *et al.*, 1999). This suggestion was supported by Chung *et al.* (1988) who reported that the addition of fresh or barely decomposed plant material such as crop residue, winter crops and weeds into the soil may have temporarily increased the risk of damping off and root diseases, since 'facultative' saprophytic pathogens such as *Pythium* spp. can multiply in this new substrate. This suggests that grapevines should not be planted directly into nursery soil after Indian mustard incorporation until the *Pythium* propagule numbers have returned to a level unlikely to cause damage. However, the effects of such treatments are likely to vary depending on soil type, soil matric potential and the activity or prevalence of *Pythium* spp. (Stephens *et al.*, 1999). The potential of mustard as a biofumigant against *Cylindrocarpon* spp. could be investigated further but the potential for damage by *Pythium* spp. should be considered.

1.21 Objectives

Objectives

The aim of this project was to investigate physical, chemical, biological and cultural control methods that seemed likely to reduce black foot disease in New Zealand grapevine nurseries and that could also be adapted for use in commercial vineyards with a view to developing an integrated management program. The objectives of this research were to:

1. Determine the efficacy of selected fungicides against three *Cylindrocarpon* spp.

- *In vitro* by assessing inhibition of mycelium growth and conidium germination.
- *In vivo*, in comparison with a biological *Trichoderma* product, as pre planting treatments to protect young plants placed into pre-inoculated field soils.

2. Evaluate the efficacy of a range of HWT temperatures and times on three *Cylindrocarpon* spp.
   - *In vitro* on mycelium growth and conidium germination
   - *In vivo* using dormant rootstock grapevines grown in a pre-inoculated site
3. Determine the physiological effects of HWT and cold storage on young grafted grapevines by assessing:
   - Subsequent plant growth
   - Water soluble carbohydrates extractions from root samples.
4. Evaluate the efficacy of three different rotation crops for reducing black foot disease incidence and further investigate the effects of different mustard treatments:
   - *In vitro* on mycelium growth and conidium germination by assessing inhibition of mycelium growth and conidium germination.
   - *In vivo*, using dormant rootstock grapevines grown in a pre-inoculated site.

NB. This research project was originally developed for a Masters degree and subsequently upgraded to a much longer PhD project. This may explain why some aspects appear to have an illogical sequence or some trials consist of illogical groups of treatments.

1.22 Novel species within the *Ilyonectria* radicicola species complex and the *Ilyonectria macrodidyma* complex

Cabral *et al*. (2012a) introduced 12 new taxa within the *Ilyonectria radicicola* (*C. destructans*) species complex which commonly occurred on six hosts, including grapevines. They used a combination of morphology, culture characteristics and multi gene analysis which included the β -tubulin, nuclear ribosomal RNA internal transcribed spacer (ITS), translation elongation factor 1-α genes and the histone H3 sequences, which were most useful. The new species were reported to be linked to previous names or taxa considered as synonyms of '*destructans'.* A second study to characterise isolates related to *Ilyonectria* species was reported by Cabral *et al.*(2012b). A similar multi gene analysis strategy (Cabral *et al.*, 2012a) revealed six monophyletic species within the *Ilyonectria macrodidyma* complex.

This recent taxonomic classification applied to the *Cylindrocarpon* fungi has clearly delineated species within the complexes and, as suggested by the authors, "will help researchers to devise control strategies"(Cabral *et al.*, 2012b). Prior to the new classifications, the current research investigated control strategies for *Cylindrocarpon* species associated with black foot disease in New Zealand. The isolates used in this research were identified by species specific PCR (Pathrose, 2012) and confidently placed within the *Ilyonectria radicicola* species complex, the *Ilyonectria macrodidyma* complex and *C. liriodendri.* The findings of this research are therefore relevant for control of *Cylindrocarpon* pathogens that cause black foot disease of grapevines.

Chapter 2

Fungicide Experiments

2.1 Introduction

Healthy plants have been shown to become infected with *Cylindrocarpon* spp. during their period of growth in outdoor nursery fields (Fourie and Halleen, 2002b; Gubler *et al.*, 2004; Halleen and Crous, 2001; Halleen *et al.*, 2003; Oliveira *et al.*, 2004), which indicates that infection occurs from soil inoculum (Halleen *et al.*, 2007). The pathogens most likely enter the pith of the rootstock cuttings through the gaps in the incomplete callusing around the basal end of the trunk and through wounds caused by the breaking off of newly developed roots during the planting process (Halleen *et al.*, 2003). Therefore, it is important to develop suitable control measures that prevent or eradicate these infections (Halleen *et al.*, 2007).

The use of chemical and biological pre-planting treatments to prevent infection has been investigated with varying results. *In vitro* experiments in Portugal by Rego *et al.* (2006) were designed to reduce both conidium germination and mycelium growth of *C. liriodendri* (originally called '*C. destructans*') which was found to be the major pathogenic species (Rego *et al.*, 2006). Azoxystrobin, trifloxystrobin and tolylfluanid inhibited conidium germination but were ineffective in inhibiting mycelium growth. Prochloraz, benomyl and carbendazim + flusilazole inhibited mycelium growth but were ineffective in inhibiting conidium germination (Rego *et al.*, 2006). Subsequent greenhouse experiments conducted with treated grapevines planted into potting mix found that benomyl, tebuconazol, carbendazim + flusilazole and cyprodinil + fludioxonil, significantly improved plant growth and decreased disease incidence of *C. liriodendri* (Rego *et al.*, 2006).

In South Africa, Halleen *et al*. (2007) tested the efficacy of fungicides in reducing mycelium growth of four black foot species, *C. liriodendri*, *C. macrodidymum*, *Campyl. fasciculare* and *Campyl. pseudofasciculare.* They found that prochloraz manganese chloride was the most effective at reducing mycelium growth of all four species while benomyl, flusilazole and imazalil were only effective in reducing mycelium growth of *C. liriodendri* and *C. macrodidymum.* In contrast, their experiments in nursery soils showed that these four fungicides as pre-planting soak treatments were not able to prevent infection by *C.*

liriodendri and *C. macrodidymum* (Halleen *et al.*, 2007). However, other more recent trials have demonstrated efficacy of these fungicides as protectant treatments for pre-planting into inoculated pots or naturally infested nursery fields. The most effective compounds were cyprodinil + fludioxonil (Nascimento *et al.*, 2007; Rego *et al.*, 2009), benomyl, Sporekill™ and captan (Fourie and Halleen, 2006).

This research aimed to fully evaluate the more effective of the fungicides in the above pre-2006 experiments and other likely compounds which were available in New Zealand, to find a reliable pre-planting method for preventing infection in nurseries and vineyards. The experiments were conducted in two stages, which are presented in separate sections of this chapter: (1) *in vitro* evaluation of fungicides for inhibiting conidium germination and mycelium growth of three identified New Zealand isolates of each of the three *Cylindrocarpon* species: *C. destructans*, *C. liriodendri* and *C. macrodidymum* and (2) field evaluation of the best of these fungicides as pre-planting protectants in soil infested with the three *Cylindrocarpon* spp. before planting.

2.2 Section 1: - *In vitro* fungicide experiments

2.3 Materials and methods

Twelve fungicides, representing nine chemical classes with different modes of action (Table 2.1) were tested. The fungicide products were prepared, according to the manufacturers' recommendations, as stock solutions in sterile distilled water (SDW) and six concentrations were prepared from these plus a control of SDW (Table 2.1).

Table 2.1 Fungicides and their concentration ranges tested for inhibition of conidium germination and mycelium growth of *Cylindrocarpon* spp.

Chemical Class/ Active ingredient	**Trade name**[1]	**Mycelium conc. range (mg a.i. /L)**	**Conidia conc. range (mg a.i. /L)**	**Grape reg.**[3]	**a.i. conc.**[2]
Anilopyrimidime Cyprodinil + Fludioxonil	Switch™ 60:40 WG	0.05 - 1.0	0.01 - 0.7	yes	375:250 g/Kg Cyp:Flu
Benzimidazole Carbendazim	MBC 500 SC	0.05 - 3.0	0.3 - 100	yes	500 g/L
Cyclic imide Captan	Captan Flo SC	10 - 150	1.0 - 15.0	yes	480 g/L
Imidazole Prochloraz	Mirage 450 EC	0.003 - 3.0	0.3 - 100	no	450 g/L
Nitrile Chlorothalonil	Chlorothalonil SC	1.0 - 20	0.3 - 100	yes	720g/L
Phenylpyrrole Fludioxonil	Maxim SC	0.05 - 3.0	0.01 - 1.0	no	100 g/L
Quartenary ammonium Didecyldimethyl-ammonium chlorine	Sporekill SC	3 - 1000	1 - 6	yes	120g/L
Strobilurin Azoxystrobin	Amistar WG	1 - 300	1 - 6	yes	500 g/Kg
Strobilurin Picoxystrobin	Acanto SC	1 - 300	0.01 - 1.0	no	250 g/litre
Triazole Difenoconazole	Score 250 EC	1 - 300	0.3 - 100	no	250 g/Kg
Triazole Flusilazole	Nustar WG	0.05 - 3.0	0.3 - 100	no	200 g/Kg
Triazole Tebuconazole	Folicur WG	1 - 300	1 - 6	no	250 g/Kg

[1] WG = Water Granule SC = Suspension EC = Emulsifiable.
[2] a.i. = Active ingredient.
[3] Registered for use on grapevines in New Zealand

The concentration range of each fungicide was determined from reports of other *in vitro* experiments and the field application rate. The concentrations differed between experiments for mycelium growth or conidium germination.

2.3.1 *Cylindrocarpon* isolates

In this study and throughout this research project (unless otherwise stated), three isolates from each of the three *Cylindrocarpon* species, which were representative of isolates collected from the grape growing regions of New Zealand (Bleach *et al.*, 2006), (Appendices A.2 and A.5), were used in all experiments (Figure 2.1). In the 2006 and 2007 Lincoln field experiments (Section 2.9 and 2.10, respectively) the same nine isolates of the three *Cylindrocarpon* species which had been used in the *in vitro* experiments (Section 2.1.2) were used. Their identity was initially determined by Mostert *et al.* (2006) and later confirmed using species specific primers (Pathrose *et al.*, 2011) as *C. destructans* (1D, 2D and 3D), *C. liriodendri* (1L, 2L and 3L) and *C. macrodidymum* (1M, 2M and 3M) (Appendix A.2). These isolates were used to provide the mixed inoculum to be tested under field conditions.

In the absence of any genetic diversity information, these isolates were selected according to different regions of isolation and colony morphologies in an attempt to represent as wide a potential genetic base as possible (Appendix A.2). All isolates were maintained on Spezieller Nährstoffarmer Agar (SNA, Appendix B.1.2) slants (Brayford, 1993) at 4°C until required. The isolates were subcultured onto potato dextrose agar (PDA; Oxoid Ltd, Basingstoke, Hampshire, England, Appendix B.1.1) plates, and incubated for 2-4 weeks when colonies had grown sufficiently to provide inoculum for the fungicide experiments. The incubation conditions for *Cylindrocarpon* spp. throughout this research programme were 20°C, with a diurnal cycle of 12 h white light and 12 h darkness, unless otherwise stated.

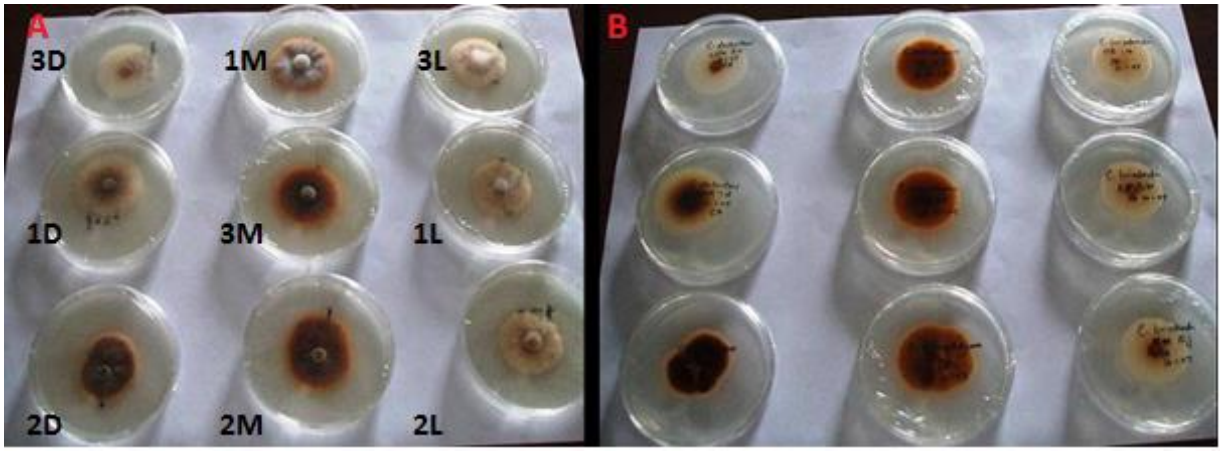


Figure 2.1 Upper (A) and underside (B) views of the three *Cylindrocarpon* species (three isolates of each *C. destructans* (3D, 1D and 2D), *C. macrodidymum* (1M, 2M and 3M), and *C. liriodendri* (3L, 1L and 2L); colonies grown on PDA plates for 14 days at 20°C.

2.3.2 Mycelium growth assay

For each fungicide, six concentrations (Table 2.1) were made by adding the appropriate amount of the fungicide stock to sterile, molten (50°C) malt extract agar (MEA, Appendix B.1.3), (Johnston and Booth, 1983) and 18 mL aliquots were dispensed immediately into individual Petri dishes. Within 1 h of pouring, each plate was inoculated centrally with one 6 mm colonised agar disc cut from the actively growing edge of a 2-4 week old culture of the *Cylindrocarpon* isolate and placed mycelium side down. There were three replicate plates for each of the nine *Cylindrocarpon* isolates, for each concentration and the untreated control of malt extract agar (MEA) only. The plates were sealed with cling film and randomly allocated to positions in a 20°C incubator for 12 days under a diurnal light schedule (12 h light, 12 h dark). Growth was assessed on each plate by measuring the perpendicular diameters with a digital calliper (Mitutoyo, UK Ltd) and the mean mycelium growth determined as a percentage of that on the control plate.

2.3.3 Conidium germination assay

This *in vitro* conidium germination experiment used the same 12 fungicides as used to assess mycelium growth (Table 2.1). The effects of the fungicides could only be investigated one fungicide at a time because assessment was a lengthy process. For each fungicide (Table 2.1), six concentrations were made with SDW to give double the required concentration which was then diluted by addition of an equal volume of the conidium suspension.

Conidium suspensions were obtained by pipetting 5 mL of SDW onto 2-4 week PDA culture plates (Section 2.3.1) and then scraping the surface of each plate with a sterile hockey stick to dislodge conidia. Each resulting suspension was poured into a separate sterile Universal bottle containing 5 mL of SDW and the conidium concentration was adjusted to $1x10^5$ conidia/mL based on haemocytometer counts. For each isolate, a 1000 µL aliquot of the suspension was mixed with 1000 µL of each fungicide concentration in a 20 mL Universal bottle to give a final conidium concentration of $5x10^4$ conidia/mL. From this suspension, three droplets (40 µL each) were pipetted 20 mm apart onto each of three glass slides which were placed onto the upper or lower surface of an 85 mm diameter Petri dish. The Petri dish was then set inside a larger square Petri dish (Figure 2.2) that contained 5 mL of water, so that it acted as a humidity chamber when covered with the lid. Each chamber contained two or three slides, which represented two or three concentrations of the same fungicide set up with one isolate. Each treatment combination was replicated three times and the replicates were randomly allocated to positions in a dark 24°C incubator where they remained for 5 h. On removal from the incubator, glass cover slips were placed onto conidium droplets. To maintain the initial germination rate while counting, the slides were kept for the 1-5 h needed in a 7-8°C temperature controlled room. Germination of 100 randomly selected conidia in each droplet was evaluated with a compound microscope at × 200 magnification. Only those conidia whose germ tubes were at least the length of a conidium were considered to have germinated (Figure 2.3). The mean germination rate from the three droplets per slide was calculated for each fungicide relative to the nil fungicide control, to calculate percent inhibition relative to the untreated control, for each replicate, isolate and fungicide concentration.

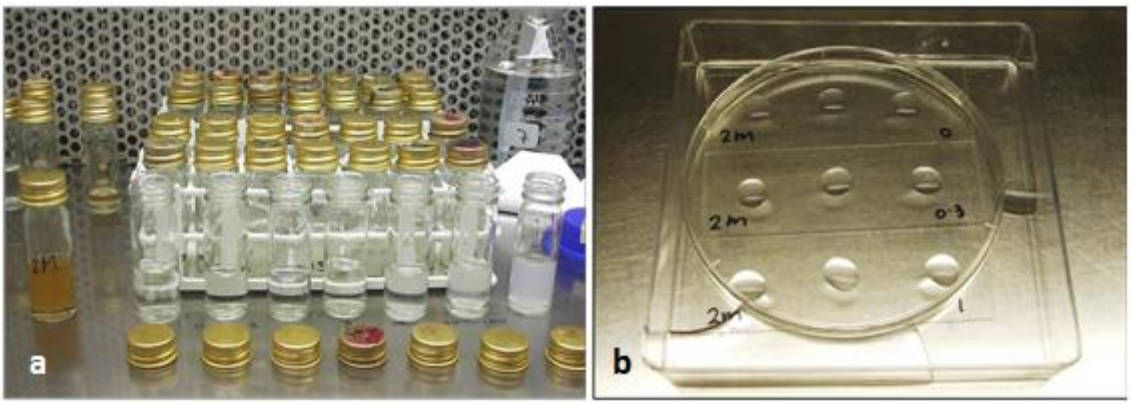


Figure 2.2 Universal bottles containing a range of fungicide concentrations to be tested a) and a humidity chamber containing three slides, each with three droplets (40 µL) of the fungicide amended conidium suspensions used to assess fungicide effects on conidium germination against *Cylindrocarpon macrodidymum* isolate 2M

2.3.4 Statistical analysis

The experimental design was a randomised factorial design with three isolates from each of the three *Cylindrocarpon* spp., 12 fungicides, seven concentrations and three replications. The data were analysed using the Probit Analysis option with SPSS version 15.0 (SPSS Inc., Chicago, USA), which calculated an EC_{50} value (effective active ingredient concentration that inhibited mycelium growth and conidium germination by 50%) from the three replicates for each isolate, and their confidence intervals (95%). When data were not suitable for this method of estimating an EC_{50}, usually because it was well outside the tested concentration range, the EC_{50} was replaced with the most relevant extreme value from the range of concentrations. To try and ensure that a realistic EC_{50} could be estimated, care was taken to select the range of concentrations which gave expected levels of efficacy as reported in the literature and so the EC_{50} values produced did account for the different concentration rates (C. Frampton, pers comm. 2011). Subsequently the EC_{50} data for mycelium and conidium inhibition, using isolates as replicates, were able to be analysed with ANOVA (GenStat version 13) to determine fungicide and species effects, and means were separated using Fisher's protected LSD at $P \leq 0.05$.

2.4 Results

2.4.1 Mycelium growth assay

The EC_{50} (mg a.i. /L) values for the effects of the various fungicides on *in vitro* mycelium growth inhibition for nine *Cylindrocarpon* isolates are presented in Table 2.2.

The mean EC_{50} value was calculated from the three isolates of each *Cylindrocarpon* species to give a mean fungicide effect for each *Cylindrocarpon* species and the LSDs used to indicate significance of differences (Table 2.3). The ANOVA of EC_{50} values (Table 2.3) showed that the overall mean fungicide effect was highly significant (P<0.001, LSD 6.790, Appendix C.3.1) as was the mean species effect (P<0.001, LSD 3.395) and the mean fungicides x species effect was also highly significant (P<0.001, LSD 11.762).

Table 2.2 Mean EC_{50} values (mg a.i./L) of 12 fungicides for mycelium growth of nine isolates in fungicide amended agar after 12 days incubation

Chemical	Fungicide Conc. range (mg a.i. /L)	Field rate (a.i.)	*Cylindrocarpon* isolates mycelium EC_{50} (mg a.i. /L)								
			* 1D	2D	3D	1L	2L	3L	1M	2M	3M
Azoxystrobin	1 - 300	0.2 g L^{-1}	40.14	11.79	38.96	>300	454.06	>300	0.23	2.88	4.40
Captan	10 - 150	0.96 g L^{-1}	47.82	28.61	50.38	72.97	73.25	42.99	50.32	84.38	71.05
Carbendazim	0.05 - 3.0	0.25 g L^{-1}	0.44	0.40	0.41	0.38	0.35	0.33	0.37	0.25	0.32
Chlorothalonil	1.0 - 20	1.5 g L^{-1}	19.96	0.65	6.69	>20	>20	6.50	<1.00	1.48	0.56
Cyprodinil + Fludioxonil 60:40	0.05 - 1.0	0.3 g L^{-1} 0.2 g L^{-1}	1.89	0.28	0.09	>1.0	>1.0	>1.0	>1.0	0.01	0.01
Didecyldimethyl-ammonium chlorine	3 - 1000	1.2 g L^{-1}	3.35	23.42	8.68	13.45	7.09	11.45	16.26	9.20	6.79
Difenoconazole	1 - 300	1.25 g L^{-1}	0.03	0.06	0.43	0.93	0.24	1.41	<1.00	0.05	0.04
Fludioxonil	0.05 - 3.0	9 g L^{-1}	0.30	0.63	19.07	6.10	10.80	>3.00	0.04	0.01	<0.05
Flusilazole	0.05 - 3.0	0.02 g L^{-1}	2.81	4.56	4.81	14.17	2.97	2.78	0.09	0.28	0.23
Picoxystrobin	1 - 300	0.8 g L^{-1}	<1.00	9.38	37.72	>300	>300	>300	2.18	<1.00	5.60
Prochloraz	0.003 - 3.0	0.25 g L^{-1}	0.06	0.06	0.04	0.05	0.02	0.04	0.02	0.02	0.01
Tebuconazole	1 - 300	0.01 g L^{-1}	9.01	4.30	0.65	3.77	1.04	6.72	<1.00	0.17	0.25

**C. destructans* (1D, 2D and 3D), *C. liriodendri* (1L, 2L and 3L) and *C. macrodidymum* (1M, 2M and 3M) (Appendix A.2).

Mycelium growth of all isolates of *C. destructans*, *C. liriodendri* and *C. macrodidymum* were inhibited by captan, tebuconazole, carbendazim, prochloraz, difenoconazole and didecyldimethyl-ammonium chlorine, with fungicidal EC_{50} values being within the ranges tested (Table 2.2). The LSDs showed that these fungicides and fludioxonil were all similar in their effectiveness to reduce mycelium growth of all three *Cylindrocarpon* species relative to the controls and that the effect of these fungicides were not significantly different, except for captan which required higher concentrations to inhibit growth. However, Fludioxonil did not inhibit mycelium growth for the three *C. liriodendri* isolates and *C. destructans* isolate 3D.

The other fungicides had different effects between species or isolates. Picoxystrobin and azoxystrobin were effective in reducing mycelium growth of *C. destructans* and *C. macrodidymum* isolates, with active ingredient EC_{50} values ranging from 0.23 to 40.14 mg/L, but were ineffective against the three *C. liriodendri* isolates, with estimated EC_{50} values of >300 mg/L, which was greater than the highest active ingredient concentration range tested of 300 mg/L. Chlorothalonil inhibited mycelium growth of only one *C. liriodendri* isolate (EC_{50} of 6.5 mg a.i. /L). Cyprodinil + fludioxonil did not inhibit mycelium growth for the three *C. liriodendri* isolates, *C. macrodidymum* isolate 1M and *C. destructans* isolate 1D Flusilazole did not inhibit mycelium growth for *C. destructans* isolates 2D and 3D, and *C. liriodendri* isolate 1L, and at low concentrations was effective against only *C. macrodidymum* isolates (Table 2.2).

Captan, although significantly different to all the fungicides, still reduced mycelium growth of all three species within the concentration range tested (10-150 mg a.i. / L), but the overall mean EC_{50} value was for a high mean concentration being 57.97 mg a.i. / L. Picoxystrobin and azoxystrobin were the least effective fungicides; they were ineffective against the *C. liriodendri* isolates and required relatively high mean concentrations to be effective against *C. destructans* isolates (Table 2.3).

Table 2.3 The mean EC_{50} values (mg a.i./L) of 12 fungicides for mycelium growth of three isolates for each *Cylindrocarpon* species on fungicide amended agar after 12 days incubation.

[4]**Fungicides x species effect**	*Cylindrocarpon* species						[2]**Mean fungicide effect**	
	C. destructans		*C. liriodendri*		*C. macrodidymum*			
Azoxystrobin	30.30	a[1]	300	b	2.50	c	110.93	e
Captan	42.27	a	63.07	b	68.58	b	57.97	d
Carbendazim	0.42	a	0.35	a	0.31	a	0.36	a
Chlorothalonil	9.10	a	15.50	b	1.02	a	8.54	bc
Cyprodinil+fludioxonil	0.75	a	1.00	a	0.34	a	0.7	a
Didecyldimethyl-ammonium chlorine	11.82	a	10.66	a	10.75	a	11.08	c
Difenoconazole	0.17	a	0.86	a	0.36	a	0.47	a
Fludioxonil	6.67	a	6.63	a	0.04	a	4.45	abc
Flusilazole	4.06	a	6.64	a	0.20	a	3.63	ab
Picoxystrobin	16.03	a	300	b	2.94	c	106.32	e
Prochloraz	0.05	a	0.04	a	0.02	a	0.03	a
Tebuconazole	4.65	a	3.84	a	0.47	a	2.99	ab
[3]Mean species effect	**10.52**	**x**	**59.05**	**y**	**7.3**	**x**		

[1]Values followed by the same letter are not significantly different according to Fisher's protected LSD.
[2]The overall fungicide effect was significant (P<0.001 LSD 6.790 (right hand column).
[3]Species effect (P<0.001 LSD 3.395), (lowest row).
[4] The fungicide across species effect for each fungicide (P<0.001 LSD 11.762) compared across the rows but not down the columns.

2.4.2 Conidium germination assay

The EC_{50} values (mg a.i./L) which indicate effects of the various fungicides on *in vitro* conidium germination of nine *Cylindrocarpon* isolates are presented in Table 2.4. Conidium germination of the three isolates each of *C. destructans*, *C. liriodendri* and *C. macrodidymum* was inhibited by picoxystrobin, azoxystrobin, captan, chlorothalonil, carbendazim, didecyldimethyl-ammonium chlorine and cyprodinil + fludioxonil, within the ranges tested. The ANOVA of EC_{50} values (Table 2.5) showed that the overall fungicide effect was highly significant (P<0.001, LSD 30.13, Appendix 3.2). The other fungicides had different effects between species or isolates (Table 2.4).

Table 2.4 Mean EC_{50} value of 12 fungicides on conidium germination of nine *Cylindrocarpon* isolates in SDW amended with fungicides after 5 h incubation.

Chemical	Fungicide Conc. range (mg a.i. / L)	Field rate	*Cylindrocarpon* isolates conidium EC_{50} (mg a.i. /L)								
			*1D	2D	3D	1L	2L	3L	1M	2M	3M
Azoxystrobin	1 to 6	0.2 g L^{-1}	2.22	2.60	0.32	5.14	0.87	0.17	4.85	0.03	1.21
Captan	1.0 - 15.0	0.96 g L^{-1}	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
Carbendazim	0.3 - 100	0.25 g L^{-1}	1.70	0.01	1.72	0.01	<0.30	0.33	0.01	<0.30	<0.30
Chlorothalonil	0.3 - 100	1.5 g L^{-1}	2.63	1.98	2.69	6.69	3.59	2.58	2.62	2.35	0.33
Cyprodinil + Fludioxonil	0.01 - 0.7	0.3 g L^{1} 0.2 g L^{-1}	0.02	0.12	0.03	0.03	0.02	0.08	0.01	0.01	0.00
Didecyldimethyl-ammonium chlorine	1 to 6	1.2 g L^{-1}	0.00	<1.00	0.02	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
Difenoconazole	0.3 - 100	1.25 g L^{-1}	33.74	16.10	1.26	8.60	11.19	318.63	72.63	0.15	0.40
Fludioxonil	01 - 1.0	9 g L^{-1}	0.08	0.05	0.10	0.07	0.04	27.62	0.00	0.01	0.00
Flusilazole	0.3 - 100	0.02 g L^{-1}	>100	>100	>100	>100	>100	>100	>100	11.04	1.92
Picoxystrobin	0.01 - 1.0	0.8 g L^{-1}	<0.01	<0.01	<0.01	0.16	0.01	0.00	<0.01	<0.01	<0.01
Prochloraz	0.3 - 100	0.25 g L^{-1}	>100	>100	>100	>100	>100	>100	>100	>100	>100
Tebuconazole	1 to 6	0.01 g L^{-1}	>6	>6	>6	>6	2.44	>6	>6	3.22	0.47

**C. destructans* (1D, 2D and 3D), *C. liriodendri* (1L, 2L and 3L) and *C. macrodidymum* (1M, 2M and 3M) (Appendix A.2).

Fludioxonil and difenoconazole inhibited conidium germination for eight of the nine isolates, both being ineffective against *C. liriodendri* isolate 3L. Flusilazole inhibited conidium germination for *C. macrodidymum* isolates 2M and 3M but not for the other seven isolates, and tebuconazole was effective against only *C. liriodendri* isolate 2L and *C. macrodidymum* isolates 2M and 3M. Prochloraz was the least effective fungicide and did not inhibit conidium germination for any of the *Cylindrocarpon* isolates at any of the concentrations tested.

The mean EC_{50} value was calculated from the three isolates of each *Cylindrocarpon* species to give a mean fungicide effect for each *Cylindrocarpon* species (Table 2.5). The mean effects were similar for the most effective fungicides azoxystrobin, captan, carbendazim, chlorothalonil, cyprodinil + fludioxonil, didecyldimethyl-ammonium chlorine, fludioxonil, picoxystrobin and tebuconazole ($P>0.05$) (Table 2.5).

Table 2.5 The mean EC_{50} values (mg a.i./L) of 12 fungicides for conidium germination of three isolates for each *Cylindrocarpon* species after 5 h incubation.

	Cylindrocarpon species				
[4]Fungicides x species effect	*C. destructans*	*C. liriodendri*	*C. macrodidymum*	**[2]Mean fungicide effect**	
Azoxystrobin	1.70	2.10	2.00	1.93	a[1]
Captan	1.00	1.00	1.00	1.00	a
Carbendazim	1.10	0.20	0.20	0.52	a
Chlorothalonil	2.40	4.30	1.80	2.83	a
Cyprodinil+fludioxonil	0.10	0.00	0.00	0.04	a
Didecyldimethyl-ammonium chlorine	0.30	1.00	1.00	0.78	a
Fludioxonil	0.10	9.20	0.00	3.11	a
Picoxystrobin	0.00	0.10	0.00	0.03	a
Tebuconazole	6.00	4.80	3.20	4.68	a
Difenoconazole	17.00	113	24.40	51.41	b
Flusilazole	100	100	37.70	79.22	bc
Prochloraz	100	100	100	100	c
[3]Mean species effect	**19.2**	**28**	**14.3**		

[1]Values followed by the same letter are not significantly different according to Fisher's protected LSD.
[2]The overall mean fungicide effect was significant (P<0.001 LSD 30.13).
[3] The species effect (lowest row).
[4]The fungicide across species effect for each fungicide were not significant (P=0.193 and P=0.531, respectively).

The lower concentrations of the tested fungicides often slowed germination, as shown for fludioxonil in Figure 2.3. Difenoconazole, flusilazole and prochloraz were the least effective fungicides respectively (51.41, 79.22 and 100 mg a.i. /L, respectively). Difenoconazole was not effective against *C. liriodendri* isolates while flusilazole was not effective against *C. liriodendri* and *C. macrodidymum* isolates and prochloraz was ineffective against all isolates of the three *Cylindrocarpon* species.

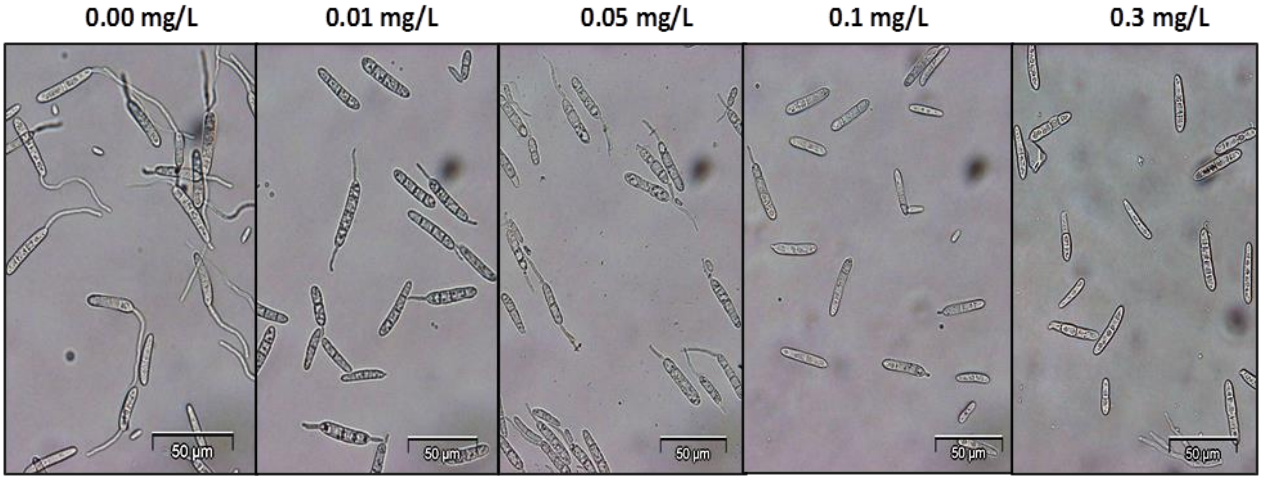


Figure 2.3 Conidial germination of *Cylindrocarpon liriodendri* isolate 2L after suspension for 5 h at 24°C in increasing concentrations (above photographs) of the fungicide fludioxonil.

2.5 Discussion

This study demonstrated the *in vitro* inhibition efficacy of fungicides belonging to different chemical classes on mycelium growth and conidium germination of three isolates each of *C. destructans*, *C. liriodendri* and *C. macrodidymum*. It is the first report of fungicide effects on inhibition of both mycelium growth and conidium germination of different isolates of the three *Cylindrocarpon* species commonly associated with black foot disease in New Zealand grapevines.

Carbendazim and cyprodinil + fludioxonil were effective in inhibiting mycelium growth and conidial germination of the three species at the lowest concentrations. Captan was effective for reducing conidium germination and mycelium growth at the lower end of the tested concentration, which reflected the generally higher field concentration used for captan compared to the other fungicides. The fungicides, prochloraz, difenoconazole, tebuconazole, fludioxonil and flusilazole were effective at inhibiting mycelium growth of the three *Cylindrocarpon* spp. but were less effective at inhibiting conidium germination. In contrast, the fungicides: azoxystrobin, picoxystrobin and didecyldimethyl-ammonium chloride, which were ineffective at inhibiting mycelia growth were highly effective at inhibiting conidium germination, although only didecyldimethyl-ammonium chlorine inhibited conidium germination for all nine isolates.

Rego *et al*. (2006) found prochloraz, carbendazim + flusilazole and cyprodinil + fludioxonil to be effective in inhibiting mycelium growth of *C. liriodendri* isolates, but tebuconazole and difenoconazole in contrast to the current study were less effective in inhibiting mycelium growth of *C. liriodendri* isolates. They also reported that of the fungicides tested, cyprodinil + fludioxonil and azoxystrobin inhibited conidium germination of *C. liriodendri* isolates, which concurs with this study, but azoxystrobin did not inhibit mycelium growth of *C. liriodendri* isolates in either study. Halleen *et al*. (2007) found prochloraz manganese chloride and flusilazole effective in reducing mycelium growth of three isolates of *C. liriodendri* and three isolates of *C. macrodidymum*. It was unclear whether there was any variation in susceptibility of the individual isolates to the fungicides as their results did not report on this and conidium germination was not tested.

Rego *et al.* (2006) tested cyprodinil on its own and found that it was not effective in inhibiting mycelium growth nor conidium germination of four *C. liriodendri* isolates with EC_{50} values of >5 mg a.i. /L. Since the mode of action of cyprodinil is generally known to inhibit hyphal growth rather than spore germination (Waechter *et al.*, 2001) its lack of efficacy against conidium germination was not unexpected but its lack of efficacy against mycelium growth was surprising. However, Rego *et al.* (2006) reported that the mixture of cyprodinil + fludioxonil was effective at inhibiting both mycelium growth and conidium germination of all the *C. liriodendri* isolates at low EC_{50} values (0.08-0.15 mg/L) but in the current study the mixture had variable efficacy against the *C. liriodendri* isolates. Since Rego *et al.* (2006) tested cyprodinil without fludioxonil their result indicated that fludioxonil may have been the effective active ingredient. The mode of action of fludioxonil, although not well understood, is thought to affect mycelium development but also to inhibit spore germination and development of the germ tube (Pillonel and Meyer, 1997).

In the current study, fludioxonil on its own was shown to be effective against most isolates, inhibiting conidium germination of the three *C. destructans* and *C. macrodidymum* isolates, and two of the *C. liriodendri* isolates. Regarding mycelium growth inhibition, fludioxonil was effective against three *C. macrodidymum* and two *C. destructans* isolates, but was ineffective against all three *C. liriodendri* isolates. This study showed that the mixture of cyprodinil + fludioxonil was effective in inhibiting mycelium growth of two isolates each of *C. destructans* and *C. macrodidymum* at the concentration range tested but was not effective for three *C.*

liriodendri isolates. This was in contrast to Rego *et al.* (2006) who found cyprodinil + fludioxonil effectively inhibited the mycelium growth of all *C. liriodendri* isolates (EC_{50} values >1.0 mg a.i. /L). The reason for the variation in effects of the fungicide mixture between the countries' isolates is not clear. The New Zealand isolates used in this study were collected from established working vineyards where cyprodinil + fludioxonil as Switch™ is routinely used to control Botrytis bunch rot (Beresford, 2005a), so the New Zealand isolates may have built up some resistance to this fungicide although this was not tested. The Portuguese isolates were collected from very young grapevines showing black foot symptoms or rooted rootstocks (Rego *et al.*, 2006) in which bunch rot had not occurred, so may not have been treated with this fungicide.

Fludioxonil was ineffective in inhibiting mycelium growth when tested against the three New Zealand *C. liriodendri* isolates but partially effective in inhibiting conidium germination. Against the four Portuguese *C. liriodendri* isolates, cyprodinil was ineffective at inhibiting mycelium growth and conidium germination (Rego *et al.*, 2006). It would appear from these results, particularly in respect of conidium inhibition, that the combined action of the two fungicides was greater than the sum of their effects individually when tested against *Cylindrocarpon* spp.

Variation in the sensitivity of the species, and isolates within species to the specific fungicides was observed in this study for both mycelium growth and conidium germination. The species which showed the greatest variation to the fungicides tested was *C. liriodendri,* with less variation between *C. destructans* and *C. macrodidymum* isolates. For the concentrations tested, only the fungicides carbendazim and prochloraz effectively inhibited mycelium growth for all nine *Cylindrocarpon* isolates, while for conidium germination, the fungicides captan, carbendazim, cyprodinil + fludioxonil, didecyldimethyl-ammonium chlorine and picoxystrobin were effective across all isolates. Variation between isolates and species was also reported by Rego *et al*. (2006) and Alaniz *et al*. (2011).

Alaniz *et al.* (2011) tested the effects of 14 fungicides for inhibition of mycelium growth and conidium germination of two isolates each of *C. liriodendri* and *C. macrodidymum*. Their results showed that carbendazim and prochloraz were effective fungicides and inhibited mycelium growth of both *Cylindrocarpon* species which agrees with the findings of this study and that of Rego *et al.* (2006). In the study by Alaniz *et al.* (2011) didecyldimethyl-

ammonium chloride inhibited mycelium growth for *C. liriodendri* isolates and azoxystrobin and flusilazole inhibited mycelium growth for *C. macrodidymum* isolates similar to the results of the current research.

Conidium germination for both *C. macrodidymum* and *C*. *liriodendri* was reported by Alaniz *et al.* (2011) to be effectively reduced by captan and didecyldimethyl-ammonium chloride, as shown in the current study. However, in both studies relatively high concentrations of captan were required for mycelium growth inhibition of all the nine New Zealand and the four Spanish *Cylindrocarpon* isolates, but a considerably reduced concentration was effective for conidium inhibition. Alaniz *et al*. (2011) concluded that in general their results were variable and that "the active ingredients that showed a good response in reducing mycelium growth did not have a good performance inhibiting conidium germination", and that "most of the active ingredients that showed a good response inhibiting conidium germination did not have a good performance reducing mycelium growth". This statement agrees with the results of the current study and that of Rego *et al.* (2006). Based on these findings it would seem practical to use a combination of fungicides that would target both propagules.

In the current research, fungicides belonging to the same chemical group were observed to have different levels of activity within the *Cylindrocarpon* species. The triazole group: difenoconazole, flusilazole and tebuconazole are also demethylation inhibitors (DMI's), as is prochloraz which was observed to have similar inhibitory activities as difenoconazole and flusilazole. The DMI fungicides disrupt sterol synthesis at a single biochemical site, which is essential for membrane structure and function, affecting mycelium growth (Beresford, 2005b). However, the different products do vary in their mechanisms of activity (Appendix C.1) (Matheron, 2001). All the triazole fungicides were highly effective in inhibiting mycelium growth of the three *Cylindrocarpon* species. However, only tebuconazole was effective in inhibiting conidium germination of one *C. liriodendri* isolate and two *C. macrodidymum* isolates, but not of the three *C. destructans* isolates. The variability of the effects of difenoconazole and tebuconazole on conidium germination in this study was not unexpected since their modes of action target mycelium growth not conidium germination, and this concurs with previous research (Alaniz *et al.*, 2011; Gramaje *et al.*, 2009; Rego *et al.*, 2006). However, flusilazole also targets germ tube growth and sporulation so it was surprising that flusilazole was ineffective in reducing germination of seven *Cylindrocarpon* isolates; it

inhibited conidium germination of only two *C. macrodidymum* isolates. This result agreed with Alaniz *et al.* (2011) who reported that flusilazole did not inhibit conidium germination of *C. liriodendri* and *C. macrodidymum* isolates in their study.

Variation in efficacy among individual active ingredients of the triazoles has been reported in other experiments, for example by Scherm *et al.* (2009) in trials with diseases of soybean (*Glycine max* (L.) Merrill). Of the 71 fungicides tested in that study, tebuconazole performed the best and difenoconazole was the least effective (Scherm *et al.*, 2009). In addition, that study also revealed that some fungicides depressed yield even though they were effective in controlling the disease, for example the combination of flusilazole + carbendazim was highly effective in reducing disease severity but yield was "below average" (Scherm *et al.*, 2009). The authors surmised that this inconsistency may have been due in part to the other fungicides being strobilurins, for which there had been widespread reports of "beneficial growth-regulatory effects" (Scherm *et al.*, 2009).

In the current study, the strobilurins, azoxystrobin and picoxystrobin were both highly effective in inhibiting conidium germination of the *Cylindrocarpon* species but they were significantly the least effective fungicides overall at inhibiting mycelium growth. Low concentrations were effective for only *C. macrodidymum* with higher concentrations being required to inhibit mycelium growth of *C. destructans* isolates, while the *C. liriodendri* isolates were not inhibited by even the highest concentration tested. This group of fungicides has a very specific mode of action which involves inhibition of mitochondrial respiration, resulting in the prevention of conidium germination and mycelium growth (Matheron, 2001). Azoxystrobin has been marketed since 1996 and is the more widely used of the two fungicides for control of soil-borne and foliar diseases of more than 120 crops, whereas picoxystrobin has only been available since 2001 and was marketed to control mainly diseases of wheat, barley and oats. Since its entry into the market it has shown "improved curative properties" in some crops compared to azoxystrobin (Uttley, 2011). Picoxystrobin is unique among the strobilurins because it shows "vapour activity and xylem mobility" to the target area of the plant as well as curative and preventative qualities (Goodwin *et al.*, 2000). These attributes are more suited to experiments on live plants therefore much of the reported research focuses on *in vivo* studies with little information being available about its effect on conidium and mycelium inhibition.

Azoxystrobin has been tested against trunk diseases of grapevines with variable results. Rego *et al.* (2006) reported that *in vitro* azoxystrobin was ineffective at inhibiting mycelium growth of four isolates of *C. liriodendri* but that it effectively inhibited conidium germination by 50% at low concentrations (≤ 5 mg a.i. / L). Against *Botryosphaeria* isolates, *in vitro* azoxystrobin failed to inhibit mycelium growth of nine different *Botryosphaeria* isolates at the highest concentration tested (1000 mg/L) and so was not included in testing for conidium germination inhibition (Amponsah *et al.*, 2012).

From the above results, fungicides were selected for further *in vivo* experiments. They included ones which were either registered for use on grapevines in New Zealand or were known to be effective against the above *Cylindrocarpon* spp. Consequently cyprodinil + fludioxonil, carbendazim + flusilazole and captan were selected to be tested in field experiments. Other possible candidates could have been tebuconazole, chlorothalonil and didecyldimethyl-ammonium chloride. However, tebuconazole was not registered for use on grapes in New Zealand so was not included. Products containing chlorothalonil, which are used to control Botrytis of grapes, have been recommended for review by the Environmental Risk Management Authority of New Zealand (ERMA) given the potential for adverse effects in humans and the environment (Robin, 2008) and so this product was excluded. Didecyldimethyl-ammonium chloride showed excellent efficacy against the *Cylindrocarpon* pathogens. It is a broad spectrum surface acting biocide and disinfectant and kills by contact action but is only active in solution since the active ingredient is inactivated quickly by organic matter. Its recommended use is as a sanitizer and so it is used in grapevine propagation practices. Since soil organic matter may have inhibited its efficacy (Lefroy Valley, 2010) it was not considered suitable for the field experiments.

2.6 Section 2: - Field experiments

2.7 Materials and methods

In 2005/06, field experiments were carried out at two commercial nurseries, Corbans Viticulture, Auckland and Pernod-Ricard, Fairhall, Blenheim. Two further experiments; one in the same year and one in the following year, were carried out at the Horticulture Research Area (HRA) of Lincoln University, Canterbury.

2.7.1 *Cylindrocarpon* isolates

To ensure that the 2006 field experiments were relevant to the range of *Cylindrocarpon* spp. found to populate New Zealand vineyard soil, the mixed inoculum was prepared from five isolates of three *Cylindrocarpon* species (Appendix A.1) which had been collected from four vineyards which had participated in the nation-wide sampling of New Zealand vineyards (Bleach *et al.*, 2006). Isolates selected had different colony morphologies and were from those regions shown to have high disease incidence, specifically Marlborough and Waipara (two isolates each) and Hawkes Bay (one isolate). These isolates were subsequently identified (Pathrose *et al.*, 2011) as one isolate each of *C. destructans* (LUPP 1132) and *C. macrodidymum* (LUPP 1047) from Marlborough, one isolate each of *C. destructans* (LUPP982) and *C. liriodendri* (LUPP986) from Waipara and an isolate of *C. liriodendri* (LUPP959) from Hawkes Bay.

In the 2006 and 2007 Lincoln field experiments (Section 2.9 and 2.10, respectively) the same nine isolates of the three *Cylindrocarpon* species which had been used in the *in vitro* experiments (Section 2.1.2) were used (Appendix A.2) to provide the mixed inoculum to be tested under field conditions.

2.7.2 Preparation of *Cylindrocarpon* inoculum

Conidium suspensions were obtained by pipetting 5 mL of SDW onto the 2-4 week PDA cultures then scraping the surface of each plate with a new glass slide to dislodge conidia. The contents of ten replicate plates for each isolate were placed in a new plastic bag and these contents were emulsified with 100 mL of SDW within a Colworth Stomacher 400, (A.J. Seward & Co., Blackfriars Rd., London, United Kingdom) at 50 Hz for 10 min (Figure 2.4a).

The resulting mash was strained through a series of sterilised sieves (pore sizes of 710, 500 and 150 µm). The mash that remained on the first sieve (710 µm) was transferred into a new stomacher bag with 100 mL of SDW and homogenised for a further 8 min then the contents were strained through a 500 µm sieve. The filtrate from the 500 µm sieve from which most of the mycelium had been removed, was strained through the 150 µm sieve and this filtrate comprised the final conidium concentration. This suspension was examined under a microscope using a haemocytometer to determine the concentration of conidia in the suspension.

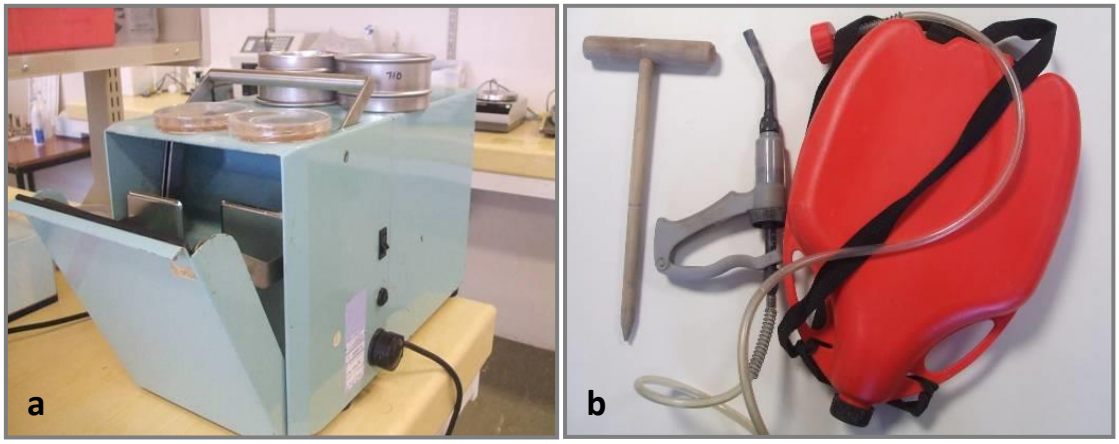


Figure 2.4 The Colworth Stomacher used to emulsify mycelium colonies (a). The stick used to make the planting hole and the drench pack and gun used to inoculate the soil (b).

This process was repeated for each of the isolates to be used in the mixed inoculum. The suspensions were observed to contain about 80% macroconidia, with the remainder being microconidia. To ensure that equivalent numbers of conidia were used, the quantities needed for the suspensions were calculated from their concentrations before mixing them. The 2 L containers of mixed isolate suspensions (~$1x10^8$/mL) were placed in polystyrene containers and packed in ice until required (within 12-24 h). When they were needed for inoculation, they were mixed with enough water to give $1x10^6$ conidia/mL. This method was used to prepare the inoculum for all field experiments. A preliminary germination test showed that germination viability of conidia from 2-4 week old cultures was 100%.

2.7.3 Rootstock material

The two node cuttings of grape rootstock varieties 101-14 Mgt. and Teleki 5C (abbreviated to 101-14 and 5C from here on) that were used in this experiment were supplied as dormant cuttings by Corbans Viticulture, Auckland and had been stored in their cool store facility (2-4°C) for 4-5 months. When the cuttings were required they were callused according to standard nursery practices for grafted vines by Corbans Viticulture staff; they were trimmed close to the basal bud, were placed upright in callusing boxes containing perlite and the boxes were placed in a callusing growth chamber at 28°C for 2-3 weeks to develop basal callus.

2.7.4 Field site preparation

Preparation of all field sites was carried out in line with standard nursery practices; the soil was cultivated, then mounded and the mounds covered in black polythene. Irrigation was not used at the Auckland site due to the moist climate. In Blenheim and in Lincoln, T-Tape drip tape, model 505 (T-Systems, Australia) was installed on the ground, under the black polythene and the sites were irrigated during the week prior to planting. Planting holes 15 cm deep were made through the polythene into the moist soil with a custom made stick (Figure 2.4b). The *Cylindrocarpon* conidium suspension was added to the bottom of the planting hole at the rate of 20 mL per hole, using a drench pack and gun (N J Phillips Pty Limited, NSW, Australia) (Figure 2.4b). A treated rootstock cutting was inserted into each hole within 2-4 h of inoculation. The rootstock cuttings were planted in double rows of six plants with 100 mm between each plant in line with standard nursery practice and the treatment plots were separated by 200 mm.

There were 12 plants per plot but only the centre eight plants of each plot were used to assess for effects of *Cylindrocarpon* spp. infection. The two treated (buffer) plants at each end of each plot were discarded, except that in Experiment 1 and 2 (Section 2.8) the buffer plants in the HWT plots and some from the inoculated control plots were HWT and grown on in a greenhouse. This was done to determine whether HWT had caused death or quiescence of the *Cylindrocarpon* spp. within the plants (Section 2.8).

2.7.5 Treatments

The fungicides selected from the experiments described in Section 1 of this chapter were carbendazim + flusilazole (Escudo®), cyprodinil + fludioxonil (Switch™) and captan (Table 2.6). The fungicides were mixed with carnauba wax (100 mL/L water) (Castle Fruit Coatings, Australia) as described by Halleen *et al.* (2007) using the application rates specified in previous publications (Nascimento *et al.*, 2007; Rego *et al.*, 2006) or the recommended soil application rate (Young, 2010). A commercial product containing conidia of *Trichoderma* spp. (10^7 cfu/mL; Tricho-Flow™, Agrimm® Technologies Limited, New Zealand) was also tested as a suspension in water (Table 2.6). Water was used to treat HWT and control plants. The bases of the grapevine cuttings (~5 cm) were soaked in their relevant treatments for 30 min as recommended (P. Fourie, pers. comm., 2005) then drained and immediately planted into the holes in their allocated plots which had previously been infested with the *Cylindrocarpon* spp. inoculum. At the Auckland and Blenheim sites two applications (January 2006 and April 2006) of the systemic foliar fungicide, FOLI-R-FOS®$_{400}$, (U.I.M. Agrochemicals (Aust) PTY LTD) were applied by nursery staff to run off at the recommended field rate (1.25 mL/L) to plants of the fungicide treatments.

Table 2.6 Fungicide treatments and application rates used for the field experiments.

Fungicide	Trade Name	Manufacturer	Concentration a.i.	Field rate
Carbendazim + flusilazole	Escudo®	DuPont	10 g L^{-1} carbendazim + 5 g L^{-1} flusilazole	2.50 mL L^{-1}
Cyprodinil + fludioxonil	Switch™	Syngenta	37.5 % cyprodinil + 25.5 % fludioxonil	1.00 g L^{-1}
Captan	Captan	Nufarm	480 g L^{-1}	1.0 g L^{-1}
Phosphoric acid	FOLI-R-FOS®$_{400}$	Agrichemicals	400 g L^{-1}	1.25 mL L^{-1}
Trichoderma spp.	Tricho-Flow™	Agrimm®	10^7 cfu/mL	10 g L^{-1}

2.8 Experiment 1: (Auckland) and 2: (Blenheim)

The Auckland and Blenheim experiments were planted in late spring 2006 using varieties 101-14 and 5C. Six treatments were planted in soil infested with the mixed *Cylindrocarpon* spp. conidium suspension and one treatment into non-inoculated soil. The treatments were (1) water for the non-inoculated control, (2) water for the inoculated control, (3) water for the HWT, (4) cyprodinil + fludioxonil in carnauba wax, (5) carbendazim + flusilazole in carnauba wax, (6) captan in carnauba wax and (7) Tricho-Flow™ (Table 2.6). The experimental design comprised six replicate blocks in which seven treatment plots, each with two different rootstock varieties, were allocated to a randomised split plot design.

The Blenheim site was irrigated in keeping with local nursery practices, irrigation being applied once each week for 2 h. Both the Auckland and Blenheim sites were managed by the on-site nursery staff according to standard nursery practices, which included a calendar spray program every 14 days of sulphur (3 kg/ha Kumulus®, BASF Canada Inc. Ontario) for powdery mildew, Dithane® (2 kg/ha, Dow AgroSciences, Indianapolis), for downy mildew and Tracel Plus (5 kg/ha Fruitfed Supplies, PGG Wrightson Limited, New Zealand) a foliar fertiliser. The vines were not trimmed at the Blenheim site but were trimmed at the Auckland site to reduce foliage which reduced plant susceptibility to mildew diseases in the moist climate (A. Graham pers comm. 2006).

The plants were grown for 8 months and when they were winter dormant, lifted from soil using a modified potato lifter which was pulled by a tractor through the soil below the root zone. The plants were removed by hand, the loose soil shaken off and the plants for assessment bundled by plot (eight plants). Immediately after lifting, the plants allocated to the HWT were washed under running tap water and were HWT at the facility at Corbans Viticulture with the assistance of their staff. The HWT followed standard nursery practices of a 30 min dip but with a slightly reduced temperature of 47°C instead of the industry standard of 50°C.

2.8.1 Plants grown post HWT

The live buffer plants from the inoculated control and HWT plots comprising 30 live plants from Auckland and 28 live plants from Blenheim were also HWT (47°C for 30 min) to determine whether the HWT caused death or quiescence of *Cylindrocarpon* spp. within the

plants. These plants were pruned to two buds and replanted in sterile potting mix and grown on for a further 8 months in a Lincoln University greenhouse, before being assessed for infection.

2.8.2 Assessment

The winter dormant plants were lifted from the Auckland site in June 2007 and the Blenheim site in July 2007. They were immediately transported to Lincoln University for assessment. Each set of eight plants, which represented one plot, was washed under running tap water and the roots and shoots removed (Figure 2.5a). From each plot the roots and shoots were treated collectively, each tissue type being placed into separate paper bags that were randomly arranged in drying ovens set at 60°C for 4 days. The dried roots and shoots were weighed one plot at a time and the weights recorded.

The bare trunks that remained (Figure 2.5b) were surface-sterilised one plot at a time by immersing for 30 s in 70% ethanol, then 5 min in 0·35% sodium hypochlorite and finally 30 s in a second solution of 70% ethanol (Halleen *et al.*, 2003). Plants that had no shoots and roots at harvest were classed as 'dead plants' but were still assessed for infection.

From each plant, the root crown was removed and discarded. A 1-2 mm section was sliced from across the basal end of the trunk (0 cm) and divided into four pieces which were placed equidistantly around the perimeter of a PDA plate amended with chloramphenicol (PDAC) (Sigma-Aldrich® Inc., MO, USA) (250 mg/L). Another 1-2 mm slice was cut from further up the trunk (5 cm) to assess the progression of the pathogen, and it was transferred to the centre of the same PDA plate. The post HWT plants were assessed in a similar way except that after the roots and shoots were removed they were discarded.

After incubation for 7 days, incidence of characteristic colonies of *Cylindrocarpon* spp. that had grown from the plant tissue was recorded (Figure 2.5c, d and e). They were allocated to species groups by colony morphology. Mycelia from these colonies were subcultured to PDA plates and incubated at 20°C for 2-4 weeks after which the plates were stored at 4°C for confirmation of identity by species specific polymerase chain reaction (PCR).

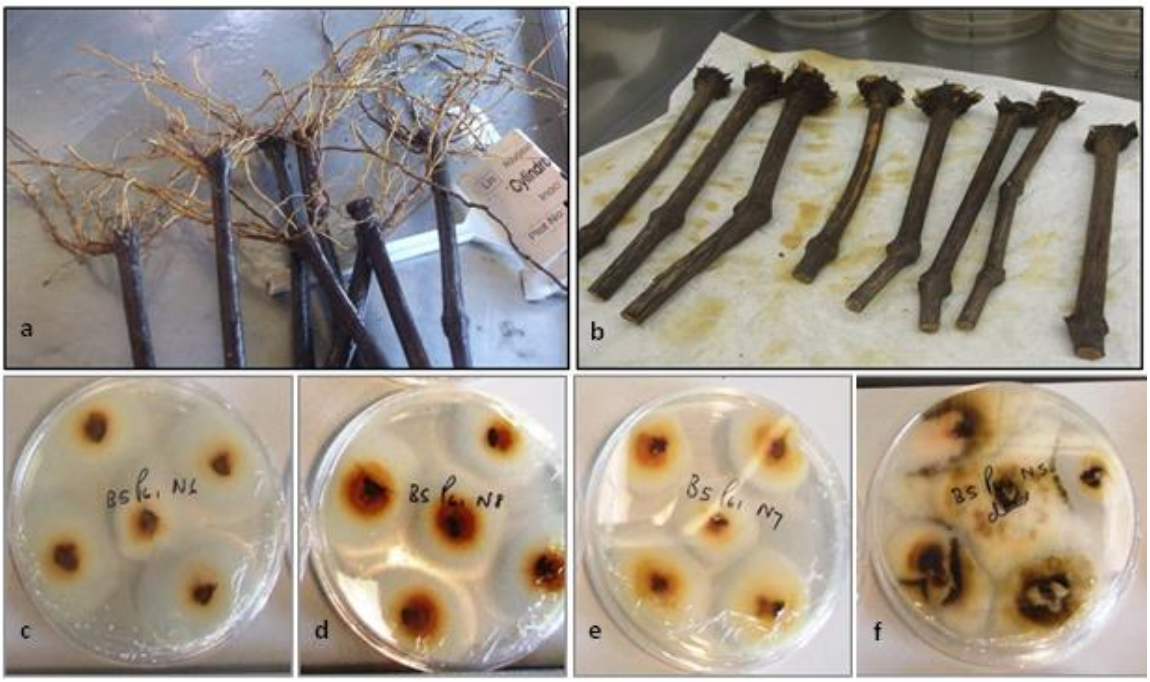


Figure 2.5 Washed rootstock with shoots removed and roots still attached (a), sterilised rootstock trunks with shoots and roots removed with the root crown still attached (b), characteristic colonies of *Cylindrocarpon* spp. that had grown from the plant tissue (c, d and e) and multiple species of saprophytic fungi grown from a dead plant (f).

2.8.3 Statistical Analysis

From each experiment the data available for analysis were the *Cylindrocarpon* spp. incidence (determined as the mean percentage of grapevine plants per plot that were infected at 0 cm and/or 5 cm) and severity (the mean proportion of tissue infection at 0 cm calculated from the four isolated segments of plants per plot), as well as the root and shoot dry weights calculated from the eight plants in each plot. This method of assessment was used for all field experiments in this study unless otherwise specified.

Analysis was carried out using a general linear model with terms appropriate to the design and the two-way interactions amongst the factors of interest (C. Frampton, pers comm. 2011). Where significant main effects or two-way interactions were identified, the significance of differences between individual treatments was further explored using standard errors or Fisher's protected LSD (least significant difference) tests. A P-value of ≤0.05 was taken to indicate statistical significance.

Post HWT plants were analysed for *Cylindrocarpon* spp. incidence (determined as the mean percentage of grapevine plants from Auckland (30) and Blenheim (28) that were infected at 0 and/or 5 cm) and severity (the mean proportion of tissue infection at 0 cm, calculated from the four isolated segments of plants, per site).

2.9 Experiment 3: Lincoln 2006

In November 2006, an experiment was set out at a Lincoln site that had not been inoculated with *Cylindrocarpon* spp. but was known to be naturally infested, since in an earlier experiment adjacent to this experimental site, control plants which had not been inoculated with *Cylindrocarpon* spp. had become infected with black foot disease (Bleach *et al.*, 2007b).

In this experiment, 101-14 callused rootstock cuttings were treated with (1) water for control, (2) cyprodinil + fludioxonil in carnauba wax, (3) carbendazim + flusilazole in carnauba wax, (4) captan in carnauba wax (Table 2.6), (5) Tricho-Flow™ (10 gL^{-1}) and (6) carnauba wax (100 mL^{-1}). The bases of the callused rootstock cuttings were soaked in the treatments for a shorter time of 5 min and then immediately planted as before. Soil preparation was the same as described in Experiment 1 and 2 and irrigation was by T-Tape drip tape applied for 30 min per day, which was controlled by an electronic timer. The treatments were arranged in a randomised block design with five replicate blocks, plots of 500 mm each with buffer zones of 200 mm between plots.

2.10 Experiment 4: Lincoln 2007

In October 2007, a second experiment was set out at a Lincoln site in the same general area but not the exact site as that for Experiment 3. In this experiment, the soil was first inoculated with a mixed conidial inoculum made from the three isolates each of the three *Cylindrocarpon* spp. used in Section 1 of this chapter, as described for Experiments 1 and 2. The experiment was arranged in a randomised split plot design using rootstock varieties 101-14 and 5C, prepared as described in Experiments 1 and 2 with five replicate blocks each containing 10 plots of 500 mm, with buffer zones of 200 mm between plots. The five treatments were: (1) water for the inoculated control, (2) cyprodinil + fludioxonil in carnauba wax, (3) carbendazim + flusilazole in carnauba wax, (4) captan in carnauba wax (Table 2.6) and (5) carnauba wax (100 mL/L). The bases of the callused rootstock cuttings were soaked

for 5 min in the treatments and then immediately planted as before. The site was irrigated as in the previous year for Experiment 3.

2.11 Molecular identification of *Cylindrocarpon* spp. isolates

After examination by conidium and colony morphology of stored cultures from the Auckland and Blenheim Experiments 1 and 2, twenty likely *Cylindrocarpon* isolates from each region, were selected for confirmation of identity by species specific PCR.
The isolates were grown on PDA at room temperature and aerial mycelium was used for DNA extraction using the REDExtract-N-Amp^{TM} Plant PCR Kit (Sigma, Missouri, United States). A tuft of mycelium (approximately 2 mm^2) was scraped from the edge of each culture using a sterile pipette tip and added to a 0.6 mL tube containing 100 µL of extraction buffer. The mixture was briefly vortexed and incubated at 95°C for 10 min. Then 100 µL of the dilution solution was added to the tube and briefly vortexed to neutralise inhibitory substances present in the extract. The mixture was centrifuged for 2 min at 3,220 x *g* and the supernatant was transferred to a new 0.6 mL tube and stored at -20°C until required.

The ribosomal DNA (rDNA) was amplified using the REDExtract-N-Amp™ PCR ready mix (Sigma, Missouri, United States) following the manufacturer's recommendations and using species specific primers Cyde small and Cyde R2 for *C. destructans* spp., both designed by Dr Hayley Ridgway (Probst, 2011), Cyma F1 and Cyma R1 for *C. macrodidymum* and Cyli F1 and Cyli R1 for *C. liriodendri* (Table 2.7) which were both provided by Dr Lizel Mostert, Stellenbosch University. To identify *C. destructans* each PCR contained 1 µL primer Cyde small (5 µM), 1 µL of primer Cyde R2 (5 µM), 4 µL of sterile nanopure water (SNW), 10 µL REDExtract-N-Amp™ PCR ready mix and 4 µL of the DNA extract. To identify *C. macrodidymum* and *C. liriodendri* the reaction mix was the same except the primers used were CymaF1/CymaR1 and CyliF1 /CyliR1 primer pairs, respectively. Negative controls using SNW instead of DNA template were included in every PCR. Samples were briefly vortexed and centrifuged at 3,220 x *g* for 5 s before placing into the PCR thermocycler (Biorad iCycler™, California, United States). The thermal cycle was performed with initial denaturation at 94^{o}C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. After amplification, 5 µL of each PCR product plus 2 µL of loading dye (Probst, 2011) was separated by electrophoresis (10 V/cm for 45 min) in a 1% agarose gel

(BIOLINE Agarose Molecular Grade, London) immersed in 1xTAE (40 mM Tris acetate, 2 mM Na_2EDTA, pH 8.5). The 1 Kb Plus DNA Ladder™ (0.1 ng/µL) (Invitrogen, California) molecular weight marker was run in the first or last lane of each gel. The agarose gels were transferred to plastic containers containing ethidium bromide (0.5 µg/mL) and allowed to stain in a shaker for 15 min, then destained in water for 10 min. The stained gels were photographed under UV light using the Versa Doc™ Imaging System Model 3000 (BIO-RAD Laboratories Inc., California).

Table 2.7 List of primers used for specific amplification of *Cylindrocarpon macrodidymum*, *C. liriodendri* and *C. destructans*.

Species	**Primer name**	**Sequence (5' to 3')**
Cylindrocarpon	Cyma F1	CTG GGA CAT GAT GGC TAA TAT GAC TTC TTG
macrodidymum	Cyma R1	GGT GGT GTG AGT TTC GTG C
Cylindrocarpon	Cyli F1	CTC CTC TTC AAC GAT CCG ACG TGC C
liriodendri	Cyli R1	GGG GCA GAG CAG ATT TCG
Cylindrocarpon	Cyde small	TGC RGG SAT TCG CTA ACG
destructans	Cyde R2	CYT GGA TAK GGG CAG ATG

2.12 Results

2.12.1 Experiment 1: Auckland

The treatments had significant effects on percent disease severity and disease incidence ($P \leq 0.001$ and $P \leq 0.001$, respectively, Appendix C.4.1 and C.4.2, respectively) in plants at 0 cm, and on disease incidence at 5 cm ($P = 0.040$, Appendix C.4.3) which were significantly reduced ($P \leq 0.05$) compared to plants within the inoculated control treatment as shown in Table 2.8.

Disease incidence at 0 and 5 cm

The HWT was most effective, causing the lowest mean incidence (14.59%) at 0 cm (Table 2.8), but was not significantly more effective than captan (26.04%). The remaining treatments were similar ($P > 0.05$, Appendix C.4.2 and C.4.3) and not significantly different to the non-inoculated control treatment. The mean percent disease incidence for plants within the inoculated control (70.84%) and the non-inoculated control (50.0%) were not

significantly different. The mean percent disease incidence at 0 cm was significantly less (P<0.001, Appendix 0) in rootstock variety 5C (36.31%) than rootstock 101-14 (50.30%), however there was no significant interaction between rootstock variety and treatment (P=0.485, Table 2.8). The treatments also affected disease incidence at 5 cm (P=0.040, Appendix C.4.3). The mean percent disease incidence at 5 cm was lowest for plants from the HWT (8.34%) and captan (10.41%) treatments however they were not significantly different (P>0.05) to plants within the non-inoculated control (15.63%) and the cyprodinil + fludioxonil (22.91%) treatments (Table 2.8).

The inoculated control had the greatest mean percentage of infected plants at 5 cm (32.29%) however this was not different (P>0.05) from the cyprodinil + fludioxonil, carbendazim + flusilazole (25%) and Tricho-Flow™ (26.04%) treatments. There was no significant effect of rootstock variety (P=0.639) on disease incidence at 5 cm nor an interaction between rootstock variety and treatments (P=0.649).

Table 2.8 Effects of treatments applied to cuttings of rootstock variety 101-14 and 5C before planting in an Auckland field experiment. Results show the mean percent disease incidence in stem bases at 0 and 5 cm and mean disease severity after 8 months growth.

Treatment (trt.) Auckland	**Incidence 0 cm mean (%)**		**Trt*Var 101-14 0 cm**	**Trt*Var 5C 0 cm**	**Severity 0 cm mean (%)**		**Trt*Var 101-14 0 cm**	**Trt*Var 5C 0 cm**	**Incidence 5 cm mean (%)**		**Trt*Var 101-14 5 cm**	**Trt*Var 5C 5 cm**
Inoculated no trt. (control)	70.84	**d**[1]	79.16	62.50	46.35	**d**	53.65	39.06	32.29	**c**	27.09	37.50
Not inoculated no trt. (control)	50.00	**cd**	56.25	43.75	23.96	**abc**	23.96	23.96	15.63	**ab**	14.59	16.66
[2]Captan	26.04	**ab**	35.41	16.66	13.80	**ab**	18.23	9.38	10.41	**ab**	14.59	6.25
[2]Carbendazim +	44.79	**bc**	45.84	43.75	30.73	**c**	32.29	29.17	25.00	**bc**	27.09	22.91
[2]Cyprodinil +	48.96	**c**	52.09	45.84	29.17	**c**	29.17	29.17	22.91	**abc**	22.91	22.91
HWT	14.59	**a**	18.75	10.41	10.16	**a**	13.02	7.29	8.34	**a**	8.34	8.34
Tricho-Flow™	47.91	**c**	64.59	31.25	28.13	**bc**	33.85	22.40	26.04	**bc**	31.25	20.84
Variety mean			**50.30**	**36.31**			**29.17**	**22.92**			**20.84**	**19.35**
Treatment	P=0.000	LSD 21.25			P=0.001	LSD 14.81			P=0.040	LSD 15.87		
Variety	P=0.001	LSD 8.12			P=0.045	LSD 6.09			P=0.639			
Treatment*Variety	P=0.485				P=0.801				P=0.649			

[1] Values within the same columns that have the same letter are not significantly

[2]Phosphoric acid (FOLI-R-FOS®400) was applied to plant foliage January and March 2007 (Table 2.6)

Disease severity

The treatment effect was significant (P<0.001; Appendix C.4.1). HWT was most effective, causing lowest mean severity (10.16%) at 0 cm (Table 2.8), but was not significantly different from captan (13.80%) or the non-inoculated control (23.96%). The remaining treatments were similar (P>0.05, Appendix C.4.1) to the non-inoculated control treatment. There was significantly greater mean disease severity for plants within the inoculated control (46.35%) than all other treatments. The mean percent disease severity at 0 cm was significantly less (P=0.045) in rootstock variety 5C (22.92%) than rootstock 101-14 (29.17%). There was no significant interaction effect between rootstock variety and treatments (P=0.801).

Dead plants

There was a significant effect of treatments on the number of dead plants at harvest (P≤0.001, Appendix C.4.4, Table 2.9). The HWT, Tricho-Flow™ and inoculated control treatments had the lowest mean numbers of dead plants (1.04, 0.0 and 1.04, respectively; P≤0.05). There were more (P>0.05) dead plants in the fungicide treatments, with the effect of cyprodinil + fludioxonil (14.59%) being less (P≤0.05) than for captan (32.29%) and carbendazim + flusilazole (32.29%). The number of dead plants of rootstock variety 101-14 and 5C was similar (P=0.201). There was no significant interaction effect between treatments and rootstock variety (P=0.073).

There was no significant effect of treatments on the number of dead plants with *Cylindrocarpon* spp. infection (P=0.140, Appendix C.4.5) however there was a significant effect (P=0.036, Appendix C.4.5) of rootstock variety on incidence of dead plants with *Cylindrocarpon* spp. infection, means being greater in rootstock 101-14 (3.63%) than in rootstock 5C (0.25%).

There was no significant effect between treatments and the rootstock varieties (P=0.567). The dead plants with *Cylindrocarpon* spp. present had high numbers of saprophytic fungi present, which often grew more quickly than the *Cylindrocarpon* spp. on the agar plates, making it difficult to isolate and identify the *Cylindrocarpon* spp. (Figure 2.5f).

Table 2.9 Effects of treatments on mean incidence of dead plants and of dead plants infected with *Cylindrocarpon* spp. of rootstocks 101-14 and 5C after 8 months growth in an Auckland field experiment.

Treatment (Trt.) Auckland	Dead plants mean (%)		Trt*Var 101-14	Trt*Var 5C	Dead with *Cylindrocarpon spp.* mean (%)	Trt*Var 101-14	Trt*Var 5C
Inoculated no trt. (control)	1.04	**a**[1]	2.09	0.00	1.04	2.09	0.00
Not inoculated no trt. (control)	2.09	**ab**	2.09	2.09	1.04	2.09	0.00
[2]Captan	32.29	**c**	33.34	18.75	4.16	8.34	0.00
[2]Carbendazim + flusilazole	32.29	**c**	27.09	37.50	6.25	10.41	2.09
[2]Cyprodinil + fludioxonil	14.59	**b**	25.00	4.16	1.04	2.09	0.00
HWT	1.04	**a**	0.00	2.09	0.00	0.00	0.00
Tricho-Flow™	0.00	**a**	0.00	0.00	0.00	0.00	0.00
Variety mean			**12.80**	**9.23**		**3.63**	**0.25**
Treatment	P=0.000		LSD 13.00		P=0.140		
Variety	P=0.201				P=0.036	LSD 0.37	
Treatment*Variety	P=0.073				P=0.567		

[1] Values within the same columns that have the same letter are not significantly different

[2]Phosphoric acid (FOLI-R-FOS®400) was applied to plant foliage January and March 2007 (Table 2.6)

Root and shoot dry weights

The treatments had significant effects on mean root dry weights (P=0.004, Appendix C.4.6, Table 2.10). Mean root dry weights were greatest in the Tricho-Flow™ treated plants (59.63 g) however these treatments had similar means as plants from the inoculated control (56.86 g) and the non-inoculated control (53.65 g) and the cyprodinil + fludioxonil (53.79 g) treatment. Mean root dry weights for plants from the captan (39.67 g), carbendazim + flusilazole (39.76 g) and the HWT (45.52 g) treatments were similar. Rootstock variety significantly affected (P=0.006) root dry weights with mean root dry weights being greater in rootstock 5C (54.10 g) than 101-14 (45.58 g). There was no significant interaction effect between treatments and rootstock variety (P=0.902).

Table 2.10 Effects of treatments applied to cuttings of rootstock variety 101-14 and 5C before planting in an Auckland field experiment. Results show the mean root and shoot dry weight (g) of plants after 8 months growth.

Treatment (Trt) Auckland	Mean root dry weight (g)		Trt.*Var 101-14	5C	Mean shoot dry weight (g)		Trt.*Var 101-14	5C
Inoculated no trt. (control)	56.86	a[1]	50.41	63.31	17.31	a	21.01	13.60
Not inoculated no trt. (control)	53.65	ab	49.61	57.69	13.28	ab	17.11	9.46
[2]Captan	39.67	c	31.53	47.81	9.70	b	7.99	11.41
[2]Carbendazim+flusilazol	39.76	c	37.30	42.23	10.30	b	12.76	7.85
[2]Cyprodinil+fludioxonil	53.79	ab	51.91	55.67	12.22	b	13.79	10.64
HWT	45.52	bc	42.72	48.32	10.79	b	14.10	7.47
Tricho-Flow™	59.63	a	55.61	63.64	16.78	a	20.17	13.39
Variety mean			**45.58**	**54.10**			**15.28**	**10.55**
Treatment	P=0.004	LSD 11.55			P=0.002	LSD 4.16		
Variety	P=0.006	LSD 5.85			P=0.000	LSD 2.42		
Treatment*Variety	P=0.902				P=0.193			

[1] Values within the same columns that have the same letter are not significantly different

[2]Phosphoric acid (FOLI-R-FOS®400) was applied to plant foliage January and March 2007 (Table 2.6)

The treatments had a significant effect on mean shoot dry weights (P≤0.001, Appendix C.4.7, Table 2.10). Mean shoot dry weights were greatest in plants from the inoculated controls (17.31 g) which were similar for the Tricho-Flow™ treated plants (16.78 g) and the plants from the non-inoculated control (13.28 g), however mean shoot dry weights did not differ significantly between the non-inoculated control and the other treatments, which had lowest mean shoot dry weights (9.7-12.22 g). There was a significant variety effect (P<0.001), with mean shoot dry weights being greater in rootstock 101-14 (15.28 g) than 5C (10.55 g). There was no significant interaction effect between treatments and rootstock variety (P=0.193).

There appeared to be an inverse relationship between root and shoot dry weights since greater root dry weights were recorded in rootstock 5C (54.10 g) than 101-14 (45.58 g) whereas greater shoot dry weights were recorded in rootstock 101-14 (15.28 g) than 5C (10.55 g).

2.12.2 Experiment 2: Blenheim

The treatments had significant effects on percent disease severity and disease incidence (P≤0.001 and P≤0.001, respectively, Appendix C.5.1and C.5.2) in plants at 0 cm, and on disease incidence at 5 cm (P<0.001, Appendix C.5.3) as shown in Table 2.11.

Table 2.11 Effects of treatments applied to cuttings of rootstock variety 101-14 and 5C before planting in a Blenheim experiment. Results show the mean percent disease incidence in stem bases at 0 and 5 cm and mean disease severity after 8 months growth.

Treatment (Trt.) Blenheim	Incidence 0 cm mean (%)		Trt*Var 101-14 0 cm	Trt*Var 5C 0 cm	Severity 0 cm mean (%)		Trt*Var 101-14 0 cm	Trt*Var 5C 0 cm	Incidence 5 cm mean (%)		Trt*Var 101-14 5 cm	Trt*Var 5C 5 cm
Inoculated no trt. (control)	58.34	**c**[1]	68.75	47.91	29.95	**b**	29.69	30.21	28.13	**c**	29.16	27.09
Not inoculated no trt. (control)	43.75	**b**	50.00	37.50	27.87	**b**	24.48	31.25	23.96	**bc**	20.84	27.09
[2]Captan	51.04	**bc**	58.34	43.75	33.85	**b**	39.06	28.65	23.96	**bc**	33.34	14.59
[2]Carbendazim + flusilazole	47.91	**bc**	54.16	41.66	22.13	**b**	20.83	23.44	30.21	**c**	41.63	18.75
[2]Cyprodinil + fludioxonil	37.50	**b**	45.84	29.16	24.48	**b**	31.25	17.71	15.63	**b**	14.63	16.63
HWT	3.13	**a**	0.00	6.25	1.30	**a**	0.00	2.60	1.04	**a**	0.00	2.09
Tricho-Flow™	39.59	**b**	37.50	41.66	23.70	**b**	18.75	30.99	19.79	**bc**	12.50	27.09
Variety mean			**44.88**	**35.41**			**23.44**	**23.22**			**21.73**	**19.05**
Treatment	P=0.000		LSD = 14.37		P=0.000		LSD = 12.03		P=0.000		LSD = 10.87	
Variety	P=0.029		LSD = 8.50		P=0.946				P=0.453			
Treatment*Variety	P=0.508				P=0.434				P=0.080			

[1] Values within the same columns that have the same letter are not significantly different

[2]Phosphoric acid (FOLI-R-FOS®$_{400}$) was applied to plant foliage January and March 2007 (Table 2.6)

Disease incidence at 0 and 5 cm

The HWT was most effective, causing lower mean disease incidence (3.13%) at 0 cm (Table 2.11) than all other treatments. Plants that were treated with cyprodinil + fludioxonil (37.5%) and Tricho-Flow™ (39.59%) and the non-inoculated control (43.75%) differed from the inoculated control (58.34%; P>0.05). The mean percent disease incidence for plants treated with carbendazim + flusilazole (47.91%) and captan (51.04%) was similar to plants of the inoculated control.

Mean disease incidence at 0 cm was significantly less (P≤0.001, Appendix C.5.2) in rootstock variety 5C (35.41%) than in rootstock 101-14 (44.88%), however there was no significant interaction between rootstock variety and treatment (P=0.508). Disease incidence at 5 cm (P≤0.001, Appendix C.5.3) followed similar trends as the effects at 0 cm except that rootstock variety was not a significant factor (P=0.453, Table 2.11).

Disease severity

Mean disease severity at 0 cm was significantly less for plants that were HWT (1.3%) compared to plants of all other treatments (Table 2.11). The remaining treatments had similar effects (P>0.05, Appendix C.5.1) and were not different to the non-inoculated control treatment. There was no significant effect of rootstock variety or its interaction with the treatments (Table 2.11).

Dead plants

The treatments did not have a significant effect (P=0.084, Appendix C.5.5) on numbers of dead plants at harvest (Table 2.12) although there were most dead plants in the captan treatment (45.84%) and least in those of the Tricho-Flow™ treatment (17.71%)

Table 2.12 Effects of treatments on mean incidence of dead plants and of dead plants infected with *Cylindrocarpon* spp. of rootstocks 101-14 and 5C after 8 months growth in a Blenheim field experiment.

Treatment (Trt.) Blenheim	**Dead plants mean (%)**	**Trt*Var**				**Dead with *Cylindrocarpon* mean (%)**		**Trt*Var**	
		101-14		**5C**				**101-14**	**5C**
Inoculated no trt. (control)	25.00	8.34	**a**	41.66	**bc**	6.25	**b**	0.00	12.50
Not inoculated no trt. (control)	33.34	4.16	**a**	62.50	**de**	9.38	**b**	2.09	16.66
[2]Captan	45.84	14.59	**a**	77.09	**e**	22.91	**a**	14.59	31.25
[2]Carbendazim+flusilazole	30.21	4.16	**a**	56.25	**cd**	8.34	**b**	2.09	14.59
[2]Cyprodinil+fludioxonil	38.54	4.16	**a**	72.91	**de**	9.38	**b**	0.00	18.75
HWT	34.38	0.00	**a**	68.75	**de**	1.04	**b**	0.00	2.09
Tricho-Flow™	17.71	0.00	**a**	35.41	**b**	3.13	**b**	0.00	6.25
Variety mean		**5.06**		**59.23**				**2.63**	**14.59**
Treatment	P=0.084					P=0.031		LSD = 12.37	
Variety	P=0.000	LSD = 7.12				P=0.000		LSD = 5.62	
Treatment*Variety	P=0.041	LSD = 19.00				P=0.697			

[1] Values within the same columns that have the same letter are not significantly different

[2]Phosphoric acid (FOLI-R-FOS®400) was applied to plant foliage January and March 2007 (Table 2.6)

Plants of rootstock 5C (Table 2.12) had significantly greater mean percent dead plants (59.23%) than rootstock 101-14 (5.06%; P≤0.05). The interaction between treatments and variety was significant (P=0.041, Appendix C.5.5), which indicated that the treatments had a more detrimental effect on rootstock 5C than on rootstock 101-14. There was a significant effect of treatment on incidence of dead plants that were infected with *Cylindrocarpon* spp. (P=0.031, Appendix C.5.6) (Table 2.12). Rootstock variety had a significant effect (P<0.001) on the incidence of dead plants with *Cylindrocarpon* spp., being greater in rootstock 5C (14.59%) than rootstock 101-14 (2.63%).

Root and shoot dry weight

The treatments had a significant effect (P=0.007, Appendix 0) on root dry weights (Table 2.13). Plants within the non-inoculated control recorded the greatest mean root dry weight (49.01 g) however this did not differ significantly from other treatments except the captan treatment (25.65 g) and the cyprodinil + fludioxonil (34.91 g).

Table 2.13 Effects of treatments applied to cuttings of rootstock variety 101-14 and 5C before planting in a Blenheim field experiment. Results show the mean root and shoot dry weight (g) of plants after 8 months growth.

Treatment (Trt.) Blenheim	**Mean root dry weight (g)**		**Trt*Var**				**Mean shoot dry weight (g)**	**Trt*Var**	
			101-14		**5C**			**101-14**	**5C**
Inoculated no trt. (control)	43.72	**ab**[1]	56.52	**c**	30.91	**b**	38.20	57.97	18.44
Not inoculated no trt. (control)	49.01	**a**	60.52	**c**	37.50	**b**	34.08	49.46	18.70
[2]Captan	25.65	**c**	41.97	**b**	9.73	**a**	35.87	58.10	13.64
[2]Carbendazim+flusilazole	38.10	**ab**	63.51	**c**	12.69	**a**	38.97	54.30	23.63
[2]Cyprodinil+fludioxonil	34.91	**bc**	59.18	**c**	10.65	**a**	35.40	54.33	16.46
HWT	37.73	**ab**	57.68	**c**	17.77	**a**	36.84	62.48	11.19
Tricho-Flow™	46.94	**a**	62.86	**c**	31.02	**b**	41.11	62.81	19.40
Variety mean			**57.41**		**21.47**			**57.06**	**17.35**
Treatment	P=0.007		LSD = 11.91				P=0.947		
Variety	P=0.000		LSD = 4.68				P=0.000	LSD = 6.33	
Treatment*Variety	P=0.014		LSD = 12.38				P=0.561		

[1] Values within the same columns that have the same letter are not significantly different (P≤0.05)

[2]Phosphoric acid (FOLI-R-FOS®$_{400}$) was applied to plant foliage January and March 2007 (Table 2.6)

Plants of rootstock 101-14 had significantly (P≤0.05, Appendix 0) greater mean root dry weight than plants of rootstock 5C (57.41 and 21.47 g, respectively). The interaction between treatment and rootstock variety (P=0.014, Appendix 0) seemed to be associated with the greater difference in root weights between varieties for plants from the fungicide and HWT; significantly lower mean root dry weights were recorded for plants of rootstock 5C which were treated with HWT (17.77 g), captan (9.73 g), cyprodinil + fludioxonil (10.65 g) and carbendazim + flusilazole (12.69 g) than for 101-14 plants from the same treatments, for which the mean root weights were 57.68, 41.97, 59.18 and 63.51 g, respectively (Table 2.12).

The treatments did not have a significant effect (P=0.947, Appendix 0) on shoot dry weights (Table 2.13) but mean shoot dry weights were affected by rootstock varieties (P<0.001). Plants of rootstock 101-14 had significantly greater mean shoot dry weight than plants of rootstock 5C (57.06 and 17.35 g, respectively) which reflected the trends for root dry weights. There was no significant interaction between variety and treatments (P=0.561) for shoot dry weights.

2.12.3 Plants grown post HWT

The HWT treatment had a similar effect on levels of *Cylindrocarpon* spp. after growth in the greenhouse as immediately after HWT. Plants from the Auckland site had low disease incidence (8.62%) and severity (4.31%), compared to the levels directly after HWT, being 14.59 and 10.16%, respectively. However, plants from the Blenheim site had 0% disease incidence and 0% severity (compared to 3.3 and 1.3%, respectively directly after HWT). *Cylindrocarpon* spp. incidence was greater in rootstock 101-14 (5.17%) than 5C (3.44%). The 58 post-HWT plants from both sites were all healthy in appearance and all plants broke bud and produced vigorous growth (Figure 2.6).

Figure 2.6 Buffer plants from the Auckland and Blenheim field experiments that were harvested, HWT and then grown on for 8 months in sterile potting mix in a Lincoln greenhouse before being assessed for *Cylindrocarpon* spp. incidence.

2.12.4 Experiment 3: Lincoln 2006

In this experiment the effects of the treatments on disease incidence and severity (Table 2.14) were not significant, probably due to the low incidence of *Cylindrocarpon* spp. recovered from harvested plants.

Disease incidence and severity

Although results were not significant (P>0.05, Table 2.14) there were trends of greater efficacy from some treatments. Plants that were treated with the carnauba wax had the least mean disease incidence and severity, (2.5 and 0.63%, respectively) which was approximately 50% less than the next best treatments for which mean disease incidence and severity were 5.0 and 1.25%, respectively, for plants treated with both cyprodinil + fludioxonil and with Tricho-Flow™. The non-treated control plants had the greatest percent disease incidence (10.0%) but plants treated with captan had the greatest disease severity (6.25%, Table 2.14).

Table 2.14 Effects of treatments applied to cuttings of rootstock variety 101-14 before planting in a Lincoln experiment on mean disease incidence and severity in stem bases at 0 cm and the mean root and shoot dry weights after 8 months growth.

Lincoln 2006 Treatment	Disease incidence mean (%)	Disease severity mean (%)	Mean root dry weight (g)		Mean shoot dry weight (g)	
Non treated control	10.00	3.75	10.50	**b**[1]	5.66	**b**
Captan	7.50	6.25	8.10	**c**	4.20	**b**
Cyprodinil+fludioxonil	5.00	1.25	13.06	**a**	8.18	**a**
Carbendazim+flusilazole	5.00	3.13	9.80	**bc**	5.08	**b**
Tricho-Flow™	5.00	1.25	9.56	**bc**	5.94	**b**
Wax	2.50	0.63	9.20	**bc**	4.36	**b**
Treatment	P =0.883	P=0.439	P=0.004	LSD 2.31	P=0.009	LSD 2.08

[1] Values within the same columns that have the same letter are not significantly different

Root and shoot dry weight

The treatments significantly affected root and shoot dry weights (P= 0.004 and P=0.009, respectively, Appendix C.6.1 and C.6.2, respectively). Plants that were treated with the fungicides cyprodinil + fludioxonil had significantly greater mean root and shoot dry weights (13.06 and 8.18 g, respectively) than plants from other treatments. The mean root and shoot dry weights for plants from all other treatments were similar and ranged from 9.20 to 10.50 g and 4.20 to 5.94 g, respectively (Table 2.14).

2.12.5 Experiment 4: Lincoln 2007

Shortly after planting this experiment in late October, a frost (-2.4°C) killed the buds and new leaves of many of the plants which resulted in stunted growth and many dead plants. Only 225 (56%) of the 400 plants to be assessed survived to harvest (May 2008) and of those plants that survived, rootstock 101-14 accounted for 57% and rootstock 5C the balance (43%). The large number of dead plants therefore, resulted in unbalanced data for the comparisons of treatments (C. Frampton, pers comm. 2011). These imbalances were allowed for in the analysis by using model generated modified marginal means (SPSS Inc., Chicago USA).

There was no significant effect of treatment on disease severity (P=0.968, Appendix C.7.1) or disease incidence (P=0.980, Appendix C.7.2, Table 2.16). Disease incidence was greatest in the inoculated control (2.01%) but was generally low for all treatments and ranged from 1.22

to 1.84 %. Disease incidence at 5 cm (results not shown) was also very low and was recorded in only six plants which were from two different plots. The treatments for those plots were captan and carbendazim + flusilazole.

Table 2.15 Effects of treatments applied to cuttings of rootstock variety 101-14 and 5C before planting in a Lincoln experiment. Results show the mean percent disease incidence in stem bases at 0 cm and mean disease severity and the mean root dry weight after 8 months growth.

Lincoln 2007 Treatment[2]	Disease incidence mean (%)	Trt*Var 101-14 (g)	5C (g)	Disease severity mean (%)	Trt*Var 101-14 (g)	5C (g)	Mean root dry weight (g)	Trt*Var 101-14 (g)	5C (g)
Non treated control	2.01	2.68	1.34	0.59	1.18	0.00	4.64	7.20	2.08
Captan	1.84	3.29	0.39	1.01	1.92	0.10	4.41	5.00	3.82
Cyprodinil+fludioxonil	1.56	1.56	1.56	0.83	0.39	1.26	7.55	8.72	6.38
Carbendazim+flusilazole	1.22	2.60	0.00	0.96	1.82	0.08	5.72	7.02	4.42
Wax	1.36	2.94	0.00	0.43	0.98	0.00	5.27	7.38	3.16
Variety mean		**2.63**	**0.58**	**0.00**	**1.24**	**0.23**		**7.06**	**3.97**
Treatment	P=0.980			P=0.968			P=0.690		
Variety	P=0.023	LSD 0.11		P=0.088			P=0.023	LSD	2.74
Treatment*Variety	P=0.688			P=0.560			P=0.868		

[1] Values within the same columns that have the same letter are not significantly different

[2] All data based on modified population marginal mean.

There was a variety effect (P=0.023, Appendix C.7.3), with plants of rootstock 101-14 and 5C having disease incidences of 2.63% and 0.58%, respectively (Table 2.16). The variety effect on disease severity, while not statistically significant (P=0.088), was due to greater severity in plants of rootstock 101-14 than 5C. Plants of rootstock 5C that were treated with carbendazim + flusilazole and with carnauba wax had zero disease incidence. In addition, disease severity was zero for wax treated plants of rootstock 5C.

Root dry weight

The treatments did not have a significant effect (P=0.690, Appendix C.7.3) on mean root dry weights. There was a significant rootstock variety effect (P=0.023) with rootstock variety 101-14 plants having greater mean root dry weights (7.06 g) than plants of rootstock 5C (3.97 g). There was a trend for increased root dry weight in plants that had been treated with the fungicide cyprodinil + fludioxonil (7.55 g), compared to the other treatments in which mean root dry weights ranged from 5.72 g for plants of carbendazim + flusilazole

treatments to 4.41 g for captan treated plants. Shoot dry weight was not measured as shoot growth had been affected by the frost and was minimal during the season.

2.12.6 Molecular identification of *Cylindrocarpon* spp. isolates

The species specific primers confirmed 24 of the 40 isolates from Experiments 1 and 2 as *C. liriodendri* (shown as black isolate numbers in Figure 2.7), and five isolates as *C. macrodidymum* (results not shown). However, the remaining 11 isolates which were neither *C. liriodendri* nor *C. macrodidymum* could not be amplified with the *C. destructans* specific primers. This may be due to the quality of the extracted DNA and the lack of optimisation of the PCR conditions for these primers using the RED Extract N Amp system. However they were later identified as *C. destructans* using species specific PCR and DNA sequencing (Pathrose *et al.*, 2011).

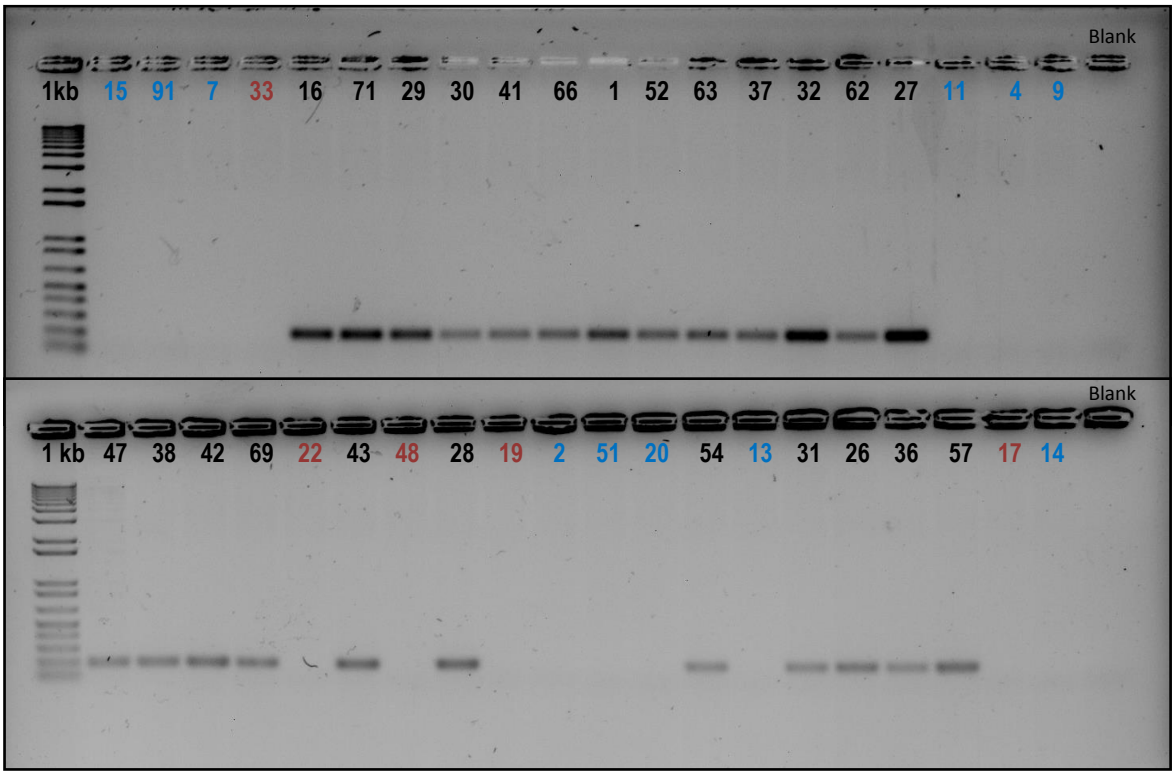


Figure 2.7 PCR products obtained with specific primers show *C. liriodendri* (black labels across the top of gel photographs and non-confirmed *C. destructans* blue labels and *C. macrodidymum* red labels). The left hand column shows the 1kb plus™ DNA ladder, and the blank: negative control on the right.

The percent of species identified from *Cylindrocarpon* isolates from the Auckland and Blenheim sites was *C. liriodendri* 65 and 55%, respectively, *C. macrodidymum* 5 and 20%, respectively and *C. destructans* 30 and 25%, respectively.

2.13 Discussion

The Auckland and Blenheim experiments, which tested the efficacy of pre-planting fungicides and *Trichoderma* (Tricho-Flow™) treatments on young plants, showed that some were effective in reducing the incidence and severity of black foot disease compared to the inoculated control, but they were less effective than the HWT, which was the significantly most effective treatment in both sites.

In Auckland, all pre-planting treatments reduced disease incidence and severity at 0 cm in comparison with the inoculated control. The captan treatment was most effective, followed by the carbendazim + flusilazole treatment, and the cyprodinil + fludioxonil and Tricho-Flow™ treatments were least effective. In Blenheim however, not all of the pre-planting treatments reduced disease in comparison with the inoculated control. The captan and carbendazim + flusilazole treatments were relatively ineffective, results being similar to the inoculated control. However the cyprodinil + fludioxonil and Tricho-Flow™ treatments had lower disease incidence at 0 cm than the inoculated control.

The lack of efficacy for the pre-planting treatments may have been affected by the high levels of inoculum added to soil prior to planting; efficacy might have been greater with lower disease pressure than used in this study. In hindsight, it appears that inoculation was unnecessary as the plants from non-inoculated treatments also had relatively high disease levels. However, it was considered important to ensure that enough plants became infected to provide for statistically valid effects, and to use all available species to fully test the fungicides.

Experiment 3 in Lincoln, which tested the effects of the fungicides, with shorter soak times, in soil with low natural *Cylindrocarpon* spp. infestation was not able to show efficacy because disease incidence and severity were very low and the results not significant. However, the cyprodinil + fludioxonil, carbendazim + flusilazole and Tricho-Flow™ treated plants all had 50% lower mean disease incidences than the non-treated control plants. The subsequent similar experiment, with inoculated soil was compromised by frost damage but

still managed to show some beneficial effects of treatment since plants of rootstock 5C that were treated with carbendazim + flusilazole or carnauba wax had zero *Cylindrocarpon* spp. incidence and zero disease severity for the wax treated plants.

Plants of rootstock 101-14 were consistently more susceptible to infection by *Cylindrocarpon* spp. than plants of rootstock 5C in the current study and this concurs with Harvey and Jaspers (2006) who reported that rootstock 5C was more tolerant to black foot disease than rootstock 101-14 in greenhouse experiments. Bleach *et al.* (2007b) also noted that in field experiments and in soils with heavy disease pressure, rootstock 5C demonstrated greater resistance to black foot infection than rootstock 101-14. Rego *et al.* (2000) also observed that rootstock 101-14 plants had higher disease incidence than rootstock SO4 (47.0 and 33.4%, respectively), and since rootstock SO4 and 5C are of the same parentage and are morphologically similar (Walker and Bourisiquot, 1992) they may be treated alike. The results from the Lincoln 2007 experiment also concurs with this, given that plants of rootstock 101-14 had significantly greater mean disease incidence than plants of rootstock 5C and disease severity while not significantly different was also greater in plants of rootstock 101-14 than 5C.

The treatments and rootstock variety had significant effects on root and shoot dry weights in all experiments. In the Auckland experiment and to a lesser extent in the Blenheim experiment, an increase in root and shoot dry weights was observed for plants treated with Tricho-Flow™, which is consistent with the general belief that it promotes growth of plants. As a rule, grapevine root:shoot dry weight ratios are similar even when vine leaves are trimmed (Buttrose, 1966, 1968). However in Auckland, rootstock 5C had the greater root dry weight and rootstock 101-104 had the greater shoot dry weight. Shoot trimming was conducted in Auckland and since rootstock 101-14 is more vigorous than 5C (Jackson, 2000) this could account for the anomaly. In the Lincoln 2006 experiment, the 101-14 plants with a cyprodinil + fludioxonil treatment had significantly greatest root and shoot dry weights and in 2007 the same treatment caused increased root dry weights of both varieties, which could have been due to reductions in levels of root disease.

At both the Auckland and Blenheim sites, plant deaths were greatest in the fungicide treatments and more dead plants were recorded from the captan treatment than any other treatment. The reasons for plant deaths were not investigated but may have been caused by

a phytotoxic effect of the fungicides, as suggested by Fourie and Halleen (2006) who reported negative growth parameters in fungicide treated plant propagation material. Regional differences in climate may have also been a contributing factor as Auckland typically has warm temperatures and high rainfall whereas Blenheim has high temperatures and low rainfall often causing droughts in summer (Cooper, 2012) which may account for the larger number of plant deaths in Blenheim than Auckland.

In the Auckland and Blenheim experiments, two phosphoric acid foliage sprays of FOLI-R-FOS® 400 were applied to the plants of the fungicide treatments in January and March as a prophylactic measure since applications of phosphoric acid had been reported to "stimulate host resistance" in plants (Schwinn and Staub, 1995) and protect plants against root pathogens such as *Phytophthora* and *Pythium* spp. (Marais and Hattingh, 1986). Foliar sprays of Fosetyl-Al, which is rapidly degraded to phosphorous acid, showed promising results in the management of esca disease of grapevines caused by Petri disease fungi and *Fomitiporia punctata* (Fr.) Murrill (Di Marco *et al.*, 2000) and was a "most effective" curative treatment of young grapevines infected with *Pa. chlamydospora* (Laukart *et al*., 2001). In the current study the efficacy of this treatment could not be assessed as the experimental design did not include a control for this treatment, but Rego *et al*. (2006) reported the positive effects of this product against *C. liriodendri* (which they called *'C. destructans'*). In that study, plants that were dipped for 50 min in a Fosetyl-Al suspension and then grown in a greenhouse for 3 months in substrate infested with *C. liriodendri* had significantly reduced disease incidence compared to untreated control plants but the product did not show *in vitro* efficacy.

In Experiments 1 and 2 (Auckland and Blenheim), carnauba wax was used in combination with the fungicide soak treatments to improve fungicide adhesion, and its sole effect was investigated in the two Lincoln experiments. Although not significant, in the Lincoln 2006 field experiment, the plants that were treated with carnauba wax and grown for 8 months had 50% less natural infection by *Cylindrocarpon*, than other treatments. In the 2007 experiment, the carnauba treated plants had lower disease incidence and severity than non-treated control plants and had greater root dry weights, although not significantly so. Halleen *et al.* (2007) reported that plants that were treated with carnauba wax and grown in field experiments had significantly greater mean root and shoot dry weights (35.1 and 28.3 g, respectively) than control plants (29.2 and 22.8 g, respectively) but that the incidence of

black foot pathogens in the carnauba wax treated plants was similar to the control plants. The reason for the slightly lower incidence in plants treated with carnauba wax was not known but it was hypothesised that the wax may have 'sealed' areas of the cuttings that were not completely callused and small wounds thereby preventing pathogen entry.

The earlier research on fungicide control of black foot disease has been completed largely by scientist groups in Portugal and South Africa. In Portugal in greenhouse experiments, Rego *et al*. (2006) reported the effectiveness of some fungicides, selected from their earlier *in vitro* experiments, on grapevine plants/graftlings grown in soil. The roots of the graftlings were trimmed and 5-10 cm of the basal ends soaked for 50 min in 14 fungicide suspensions, prepared according to recommended field rates, before being grown in pots of an autoclaved medium of soil, peat and sand which was infested with four *C. liriodendri* isolates (identified as *C. destructans*) for three months and then uprooted and assessed. They reported that benomyl, tebuconazole, carbendazim + flusilazole and cyprodinil + fludioxonil "significantly improved plant growth and decreased disease incidence" compared to control plants. Nascimento *et al*. (2007) conducted a similar experiment on potted grapevines in a greenhouse. Plant roots were trimmed and the basal ends soaked in three fungicide treatments for 50 min prior to planting in potting mix which had been artificially infested with *Pa. chlamydospora* or *C. liriodendri*. A chitosan foliar spray was included as another treatment. After three months growth, the incidence of *C. liriodendri* in the uprooted vines was lowest in the plants treated with cyprodinil + fludioxonil (21.67%) and chitosan (32.05%), being significantly less than for the control (80.83%), which was not significantly different to the tebuconazole (43.33%) and carbendazim + flusilazole (40.83%) treatments. A similar trend was reported with *Pa. chlamydospora* isolations although the percentage of isolations overall were much less with cyprodinil + fludioxonil (3.7%) and chitosan (3.3%) compared to the control (8.3%) which was similar to tebuconazole (6.7%) and carbendazim + flusilazole (11.7%) (Nascimento *et al.*, 2007).

In South Africa, field experiments were carried out by Halleen *et al*. (2007) at two nurseries during the 2003 and 2004 seasons to test biological treatments comprising *Trichoderma* spp. and different adjuvants mixed with one of four fungicides, which had been found effective in earlier *in vitro* experiments. The basal ends (5-10 cm) of the callused rootstock cuttings were dipped for 1 min in one of the suspensions which were: benomyl, benomyl/carnauba wax or

benomyl/Nu-Film 17, flusilazole, flusilazole/carnauba wax or flusilazole/Nu-Film 17, imazalil, prochloraz manganese chloride, prochloraz manganese chloride/carnauba wax or prochloraz manganese chloride/Nu-Film 17, Trichoflow-T™ (Agrimm® Technologies Limited, Christchurch, New Zealand) and a water control. Additional treatments were soil amendments with products containing *Trichoderma* spp.; Trichopel -R™ was added to planting furrows before planting and Trichogrow™ a root zone drench, was applied six times after planting. Hot water treatment (50°C for 30 min) was also applied to dormant young vines after harvest. The treated plants were grown for seven months in soil known to be naturally infested with the black foot and Petri disease pathogens.

The results showed that in both seasons the chemical or biological treatments did not significantly reduce the incidences of black foot pathogens nor improve plant growth in the rooted cuttings, however, the HWT was highly successful. Halleen *et al.* (2007) reported that apart from the HWT, the inconsistent results of the treatments in preventing infection by black foot disease pathogens may have been due to the generally low and varying infection levels in the roots and rootstocks. This is a risk of using naturally infested sites and proved to be so in the Lincoln 2006 fungicide experiment, however inoculating a site with a target pathogen may also lead to abnormally high disease pressure, which may not be controlled by the treatments, which appeared to have been an influencing factor in the results of the Auckland and Blenheim experiments. In a more recent experiment, Alaniz *et al*. (2011) tested the efficacy of seven fungicides in preventing infections by two isolates each of *C. liriodendri* and *C. macrodidymum*. The cuttings were planted into infested non-soil substrate and immediately irrigated with 20 mL of the fungicide treatment and grown for one month before root disease severity and incidence were assessed. Six of the fungicides, captan, carbendazim, copper oxychloride, prochloraz, didecyldimethyl-ammonium chloride and hydroxyquinoline sulphate, significantly “decreased the root disease severity values” for both *Cylindrocarpon* species compared with the control treatment. However, disease incidence for cuttings inoculated with *C. liriodendri* was significantly reduced by only captan, carbendazim and didecyldimethyl-ammonium chloride, while for cuttings inoculated with *C. macrodidymum* spp. only prochloraz reduced disease incidence (Alaniz *et al.*, 2011). Comparison with the above greenhouse experiments have demonstrated different effects between pot experiments with non-soil media and those conducted in natural soil.

In contrast to the effects of this study and the South African studies, Rego *et al.* (2009) reported efficacy of a pre-grafting soak of cyprodinil + fludioxonil in significantly reducing the incidence and severity of *Cylindrocarpon* spp. in two field sites, one without a grapevine history and one used as a grapevine nursery for many years with a known history of *Cylindrocarpon* infection. Prior to grafting, the dormant grafted plants were soaked for 50 min in commercial formulations of cyprodinil + fludioxonil (same as Switch™; 1.00 g/L) and fludioxonil (1.00 g/L) or cyprodinil (1.00 g/L or water for the control. After grafting, the plants were callused for three weeks then planted into the two field nurseries and grown for nine months before disease incidence and severity were assessed. Their results showed that the pre-grafting soak in cyprodinil + fludioxonil, but not fludioxonil or cyprodinil alone, had significantly less *Cylindrocarpon* incidence and severity in one field site (10 and 0.83%, respectively) than control plants (40 and 11.62%, respectively). In the site with a previous grapevine history, plants treated with cyprodinil + fludioxonil and fludioxonil alone also had significantly less disease incidence (40 and 25%, respectively) and severity (4.17 and 2.92%, respectively) than the water treated control plants (75 and 10.42%, respectively). The mixture of cyprodinil + fludioxonil also significantly decreased the number of plants that were infected with *Botryosphaeriaceae* and *Phomopsis* spp. in both the field nursery without and with a grapevine history (25 and 50%, respectively) compared to control plants (70 and 85%, respectively) (Rego *et al.*, 2009).

Since *Botryosphaeriaceae* and *Phomopsis* spp. are likely to be present in or on the cuttings used for grafting, the efficacy of the pre-grafting soaks is not unexpected. However, it is difficult to understand the mechanisms of protection which prevented the infection of plants soaked three weeks before placement into infested soils. The degradation half-life of cyprodinil in soil is 0.1-2 days and fludioxonil is 14 days (Syngenta, 2010) which may account for the better efficacy of fludioxonil than cyprodinil in their study. However, the pre-grafting soak treatment used by Rego *et al.* (2009) may have allowed for better penetration than a pre-planting treatment, since Halleen *et al.* (2007) concluded that poor efficacy of fungicides in soil could have been caused by their inability to penetrate the xylem tissue of rootstock plants or that they degrade too quickly on the rootstock surface to provide any long term protection to the plant against soil borne pathogens like *Cylindrocarpon* spp. Furthermore fungicides have been shown to have less persistence when added directly to soil than when applied to plant material as a surface treatment (Griffith and Matthews, 1969). Griffith and

Matthews (1969) reported that when captan and thiram were added directly to soil, the fungicides' half-lives were 1-2 days, which increased to 21 days, with nearly 100% initial concentrations, when coated onto the surface of glass beads (to simulate coated seeds).

The greater potential for grapevines to become infected with black foot disease when planted into a site with a previous grapevine history study, by Rego *et al*. (2009), indicated the survival of propagules in the soil. Conidia of *Cylindrocarpon* spp. were reported to have survived in normal soil without organic material as dormant chlamydospores (Taylor, 1964) but as conidia might eventually disappear in the absence of suitable substrata for growth. Probst (2011) reported that all types of propagules were able to infect grapevines grown in potting mix, however the pathogenicity of conidia, chlamydospores and mycelium-infested organic material varied between different soils. The current study concurred with the findings of Rego *et al.* (2009) since it also showed that the native black foot pathogens in the non-inoculated Auckland and Blenheim nursery soils were able to colonise the rootstock cuttings and, their ability to infect grapevines regardless of soil type. The current study also concurs with Probst (2011), who confirmed the pathogenicity of the three *Cylindrocarpon* spp. found in New Zealand vineyard soils, since the identity of *Cylindrocarpon* isolates collected from infected plants from both the Auckland and Blenheim sites was confirmed for two of the three *Cylindrocarpon* spp. by PCR and *C. destructans* later by Pathrose *et al.* (2011).

The most effective treatment in this study was HWT of the dormant plants with high rates of infection. Although the effectiveness of HWT in eliminating or greatly reducing decline organisms has been demonstrated for nursery material (Fourie and Halleen, 2006; Graham, 2007a; Graham, 2007b; Halleen *et al.*, 2007) anecdotal evidence from nursery representatives has indicated that there may be problems with survival rates of treated grapevine nursery material using the industry standard HWT of 50°C for 30 min. Waite and Morton (2007) and Graham (2007) suggested that plant tolerance to standard HWT may be affected by the temperatures in the climate of growth, indicating that cuttings grown in New Zealand's cool climate and their pathogens may be more susceptible to a reduced HWT temperature than the industry standard. In addition, Armengol *et al*. (2007) reported that cuttings and their pathogens grown in warm climates like Spain, were less susceptible to HWT and that temperatures as high as 53°C might be required to eliminate *Pa.*

chlamydospora and *P. aleophilum* within plant materials. There have been many studies undertaken into the control of pathogens of grapevine material using an increased HWT temperature as most of those studies were undertaken in warmer viticultural regions such as South Africa, (Crous *et al.*, 2001; Fourie and Halleen, 2002a; Fourie and Halleen, 2004; Fourie and Halleen, 2006; Halleen *et al.*, 2007), Spain (Armengol *et al.*, 2007; Gramaje *et al.*, 2008; Gramaje *et al.*, 2010), California (Rooney and Gubler, 2001) and Australia, (Crocker *et al.*, 2002; Edwards *et al.*, 2004; Laukart *et al.*, 2001) but little evidence of the effectiveness of reduced HWT temperatures.

The current study used a reduced hot water temperature (47°C for 30 min), as recommended by Graham (2007). Her results showed that pathogen incidence differed between two geographically different regions of New Zealand, being 14.59% for Auckland plants and 3.13% for Blenheim plants. However, the reduced temperature treatment which was 95% effective against *Pa. chlamydospora* (Graham, 2007) was clearly not as effective on *Cylindrocarpon* spp. and so further research is needed to establish the most effective protocol for eliminating all *Cylindrocarpon* spp. within dormant infected plants from New Zealand nurseries. Still, when the 'post HWT' treated plants were grown on for eight months in the greenhouse they had a similar low level of infection to those plants which were assessed immediately after HWT. This indicated that the treatment had killed the pathogens and not caused them to become quiescent within the plants. In addition, the HWT did not appear to have a negative effect on the plants since they all thrived.

In summary, this is the first study to investigate control of the three *Cylindrocarpon* species associated with black foot disease, *C. liriodendri*, *C. macrodidymum* and *C. destructans*, in New Zealand vineyard soils. Previous research was limited to a few isolates of C*. liriodendri* and to a lesser extent *C. macrodidymum* under controlled conditions or in soils with low native inoculum (Fourie *et al.*, 2006; Fourie and Halleen, 2006; Gramaje *et al.*, 2010; Halleen *et al.*, 2004a; Nascimento *et al.*, 2007; Rego *et al.*, 2006; Rego *et al.*, 2009). The current *in vitro* study established that there was variability between the *Cylindrocarpon* species and isolates within each species. It also showed that isolates of *C. liriodendri* were relatively more resistant to the fungicides than isolates of *C. macrodidymum* and *C. destructans* and that not all fungicides tested were effective against mycelium and conidia. However, the field trials have demonstrated that efficacy of the fungicides was affected by inoculum

concentrations and the soil environment. Clearly these fungicides need further investigations, possibly with long pre-grafting soaks as well as pre-planting dips. The efficacy of fungicides may prove to be site specific. The most effective treatment against the black foot pathogens was the modified HWT of 47°C for 30 min as it significantly reduced the infection levels. As the HWT did not completely rid the plants of black foot pathogens, further investigations will be conducted to establish more effective HWT protocols.

Chapter 3

Hot water treatment to reduce *Cylindrocarpon* spp. infection of young plants

3.1 Introduction

Grapevine nurseries often carry out hot water treatment (HWT) of propagation material on one year old vines as a prophylactic treatment to rid grapevines of exogenous and endogenous fungal, viral and bacterial pathogens. The 'industry standard' is to immerse grapevine cuttings or dormant rootlings (young grafted rooted vines) in a hot water bath of 50°C for 30 min and then a cold water bath for an equivalent time to minimise heat damage to the vines (Waite and Morton, 2007). After HWT the cuttings or rootlings are either cool stored or rootlings may be immediately distributed to clients to be planted into vineyards.

Research has shown the effectiveness of HWT to rid grapevine propagation material of pests and pathogens (Armengol *et al.*, 2007; Crous *et al.*, 2001; Halleen *et al.*, 2004a; Rooney and Gubler, 2001; Waite and Morton, 2007), but anecdotal reports have suggested that HWT may cause damage and so affect the viability of treated plant material (Ciancio *et al.*, 2008). However, grapevines grown in warmer climates have been reported to accumulate heat shock proteins which provide increased thermotolerance to dormant grape buds (Morrell *et al.*, 1997) thereby protecting them from some negative effects of HWT. Vines grown in cooler climates, like New Zealand, appear to be less heat tolerant, since many HWT canes failed to break bud by six weeks after grafting and planting. However, most other anecdotal reports do not cite such high rates of losses unless the HWT or post-HWT storage was mismanaged (H. Waite, pers. com. 2007). In Chapter 2, HWT at 47°C for 30 min was shown to significantly reduce incidence of *Cylindrocarpon* spp. infection but not all infections were eliminated by HWT from the dormant grapevine rootlings. By developing protocols more suitable to New Zealand's cool climate, i.e. a temperature slightly higher than 47°C but less than 50°C, black foot infection may be reduced further and the putative negative effects of HWT minimised.

Section 1 of this chapter investigated the *in vitro* efficacy of HWT for inhibiting subsequent mycelium growth and conidium germination of *Cylindrocarpon* spp. using a number of HWT temperature and time combinations. An *in vitro* trunk inoculation experiment using rootstock cuttings investigated the insulating effect of the wood on the pathogen within the trunks to HWT. Section 2 investigated the hot water treatments that were considered most likely to be successful at eliminating *Cylindrocarpon* spp. The HWT was carried out on grapevines that had been grown in a commercial nursery in Auckland, in soil that had been infested with *Cylindrocarpon* spp. Both the *in vitro* and *in vivo* experiments used the same nine *Cylindrocarpon* isolates that were used in Sections 2.2 and 2.10.

3.2 Section 1: - *In vitro* hot water treatment experiments

3.3 Materials and methods

3.3.1 *Cylindrocarpon* isolates

The nine *Cylindrocarpon* isolates of *C. destructans* (1D, 2D and 3D), *C. liriodendri* (1L, 2L and 3L) and *C. macrodidymum* (1M, 2M and 3M) were maintained on SNA slants at 4°C as described in Chapter 2 (Section 2.3.1). The isolates were subcultured to PDA plates and incubated at 20°C for 2-4 weeks when colonies had grown sufficiently to provide inoculum for the HWT experiments.

3.3.2 Mycelium plugs

Mycelium plugs of 3 mm diameter were cut from the growing edges of the 2-4 week old colonies for each of the nine *Cylindrocarpon* isolates (Section 2.3.2). Three replicate agar plugs for each isolate and for each temperature/time combination, were each placed into a separate 0.6 mL tube containing 100 µL of SDW and the lid closed. The 27 tubes for each temperature time treatment of the nine isolates were then randomly arranged in a PCR temperature cycler, an Eppendorf Mastercycler® (Biorad iCyclerTM, California, United States) and treated together. The treatments in the Eppendorf Mastercycler® were 40, 45, 47, 50 and 55°C for 5, 15 and 30 min. The control tubes were held at ambient room temperature of 20°C ± 2°C for 30 min. The Eppendorf Mastercycler® automatically dropped the temperature to 4°C for a holding period of ~3-5 min and then the tubes were removed. Each mycelium plug was removed from its tube and immediately placed onto a MEA plate.

The plates were sealed with cling film and randomly allocated to positions in a 20°C incubator for 12 days under a diurnal light schedule (12 h light, 12 h dark). Growth was assessed on each plate at day 7 and day 12 by measuring the perpendicular diameters with a digital calliper, and the mean mycelium growth was determined as a percentage of the untreated controls (Section 2.3.2).

3.3.3 Conidium suspension

Conidium suspensions were prepared for each of the nine *Cylindrocarpon* isolates by pipetting 5 mL of SDW onto 2-4 week PDA culture plates as described in Chapter 2 (Section 2.3.3) and then scraping the surface of each plate with a sterile hockey stick to dislodge the conidia. Each resulting suspension was poured into a separate sterile Universal bottle containing 5 mL of SDW and the conidium concentration was adjusted to $1x10^4$ conidia/mL using a haemocytometer. For each isolate and for each temperature/time combination, 120 µL of the spore suspension was placed into a 0.6 mL tube and the lid closed. The three replicates per treatment combination, comprising 27 tubes for each temperature/time treatment, were randomly arranged in the Eppendorf Mastercycler® for each of the following treatments, 40, 45, 47 and 50°C for 5, 15 and 30 min. The untreated control tubes were held at an ambient room temperature of 20°C ± 2°C for 30 min. The Eppendorf Mastercycler® automatically dropped the temperature to 4°C for a holding period of ~3-5 min and then the tubes were removed.

On removal from the Mastercycler® three 40 µL droplets of the conidium suspension in each tube were pipetted onto a glass slide. The glass slides were placed in humidity chambers as described in Chapter 2, and these randomly allocated to positions in a 25°C incubator, where they were incubated in the dark for 5 h. The higher incubator temperature was used for the conidium assay to induce conidia germination within a 5 h period so that germination could be assessed on the same day that the experiment was conducted.

To maintain the 5 h germination rate, and prevent further development during the assessment period, glass cover slips were placed onto the conidium droplets on removal from the incubator and the slides were then placed for 3-4 h in a 7-8°C temperature controlled room until counting was completed. Germination of 100 randomly selected conidia in each droplet was evaluated with a compound microscope at x 200 magnification.

Only those conidia whose germ tubes were at least the length of a conidium were considered to have germinated. The average of the three readings for each tube and temperature/time combination was recorded from the three droplets and the mean percent germinated conidia determined relative to the untreated controls.

3.3.4 Conidium viability test

The HWT temperature/time combinations of 40, 45 and 47°C for 15 min resulted in zero conidium germination after 5 h so, to confirm that germination was not simply delayed, the cover slips were removed and the glass slides were returned to the moist chambers and randomly placed in the 25°C incubator for a further 24 h. After this time, the slides containing the HWT spore suspension for the nine *Cylindrocarpon* isolates were assessed for conidium germination.

3.3.5 Trunk inoculation

During winter, the dormant shoots grown during the last year on 101-14 rootstock mother vines were removed and cut into 40 cm pieces to provide the canes for this experiment. The canes were surface sterilised (Section 2.8.2) and left to air dry in the laminar flow cabinet. Once dry, the canes were clamped into a bench vice ('P&B Made in England') and an electric drill with a 2 mm bit (Makita™ New Zealand Limited) which had been surface sterilised with 70% ethanol and flamed, was used to drill three holes into the pith of each cane approximately 80 mm apart, (Figure 3.1a) to provide inoculation ports for the *Cylindrocarpon* isolates.

For each of the nine *Cylindrocarpon* isolates a 3 mm mycelium plug was cut from the growing edges of the 2-4 week old colonies (Section 3.3.2) and placed into a hole in each cane using aseptic techniques in a laminar flow cabinet. There were three replicate canes for each of the nine *Cylindrocarpon* isolates and temperature treatments. To avoid pseudo-replication the three holes within a cane were inoculated with a different *Cylindrocarpon* spp. (Figure 3.1c). The cavity was filled with sterilised sawdust which was prepared earlier by collecting sawdust from holes drilled into surplus canes and autoclaving it (121°C for 15 min). The mycelium plug and sterilised sawdust were held in place by wrapping the inoculation area with a thin layer of waterproof grafting tape (Aglis & Co., Ltd Fukuoka, Japan) (Figure 3.1b).

The inoculated canes were placed into a clean plastic bag and randomly allocated to positions in a 25°C incubator for 7 d in the dark. After this, the cane pieces were removed from the incubator and HWT by placing them for 30 min in a water bath set at 47, 48.5 or 50°C. After HWT, the canes were immediately plunged into cold water for 30 min. The control canes were inoculated in a similar manner but they were not HWT. The canes were air dried in a laminar flow cabinet before the grafting tape was removed (Figure 3.1b). From the inoculation point (0 cm) and ~1 cm above and below that point, a 1-2 mm section was sliced across the canes (Figure 3.1c) and divided into four pieces. These tissue pieces were then placed equidistantly around the perimeter of a PDAC plate (Figure 3.1d) and incubated for 7 d (Section 2.8.1). The data available for analysis were the presence or absence of the inoculating *Cylindrocarpon* spp. (incidence) in each cane at 0 cm and ~1 cm above and below the inoculation point.

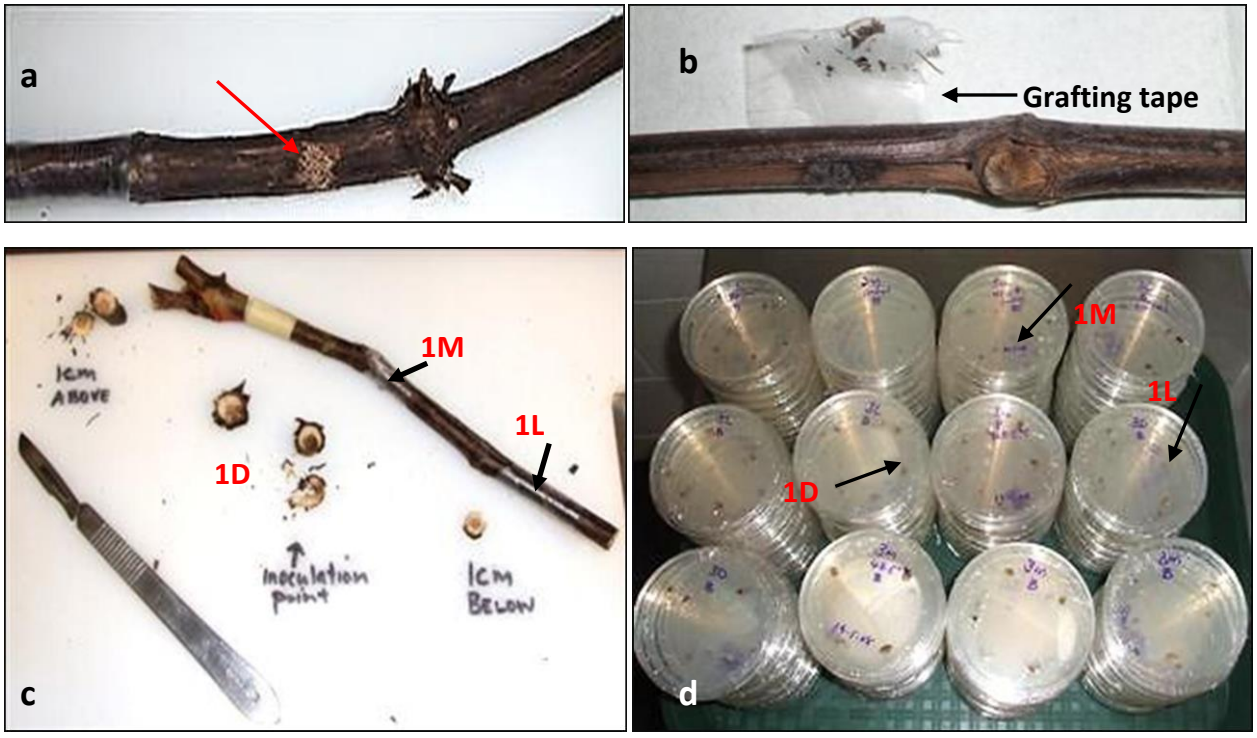


Figure 3.1 An inoculated rootstock cane, which had the cavity filled with a mycelium/agar plug and sterilised sawdust before HWT (a). Post HWT and 12 days incubation, the grafting tape was removed from the inoculation point to assess disease development (b). A HWT rootstock cane with one of the three inoculated sections removed and cut into three sections: inoculation point and 1 cm above and below (c). Pieces of rootstock tissue on PDAC ready to be incubated (d).

3.3.6 Statistical Analysis

The experimental design was a randomised factorial design with nine isolates, (three isolates from each of the three *Cylindrocarpon* spp.) and three replications for each of the experimental combinations. The data were analysed by general analysis of variance using SPSS version 17.0 (SPSS Inc., Chicago, USA) to determine HWT temperature/time and species effects, and when significant effects occurred, means were separated using Fisher's protected LSD at P≤0.05.

3.4 Results

3.4.1 Mycelium plugs

There was a significant effect of species (P=0.034, LSD=4.60, Appendix D.1.1) and isolates (P≤0.003, LSD=6.82, Appendix D.1.1) within species. Mycelium growth of *C. liriodendri* was significantly less inhibited by the temperature treatments than *C. macrodidymum* but neither was different to *C. destructans* (53.7, 59.6 and 58.0%, respectively, of the untreated control growths, LSD=4.60). However, these trends were not always consistent across the isolates within a species. Isolates 1M and 3M of *C. macrodidymum* and 2D of *C. destructans* were significantly more inhibited in their mycelium growth than *C. liriodendri* isolate 3L (P≤0.05, LSD=6.82), which was least affected by the HWT (Figure 3.2).

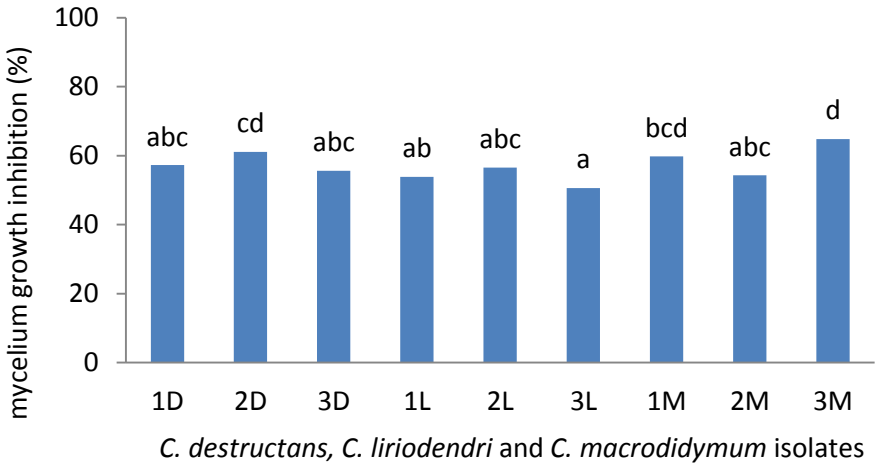


Figure 3.2 The percent mycelium inhibition for nine *Cylindrocarpon* isolates after hot water treatment (HWT), showing overall means for temperature/time combinations of 40, 45, 47, 50 and 55°C for 5, 15 and 30 min. Bars with the same letter are not significantly different according to Fisher's protected LSD (P<0.001, LSD=6.82).

However, growth of 3L was not significantly different to growth of isolates 1L and 2L of the same species and isolates 1D and 3D of *C. destructans* and 2M of *C. macrodidymum* (Figure 3.2). The interaction between species, temperature and time was not significant (P=0.362).

The HWT temperatures significantly affected mycelium growth of isolates overall (P<0.001, Appendix D.1.1), with inhibition increasing due to increasing temperature, means being 0.3, 43.4, 64.3, 79.5 and 98.2%, respectively for 40, 45, 47, 50 and 50°C (P≤0.05, LSD=5.08). Similarly, HWT time had a significant effect on mycelium growth (P≤0.001, Appendix D.1.1), with significantly greater inhibition as the HWT time increased, being 30.9, 62.2 and 78.2% for 5, 15 and 30 min, respectively, (LSD=3.94).

There was a significant interaction between HWT temperature and time (P<0.001, D.1.1) for mycelium inhibition of isolates overall (P≤0.05, LSD=8.8) (Figure 3.3). There was no significant difference between treatment times at the lowest HWT temperature of 40°C for 5, 15 and 30 min, and 5 min at 45°C (inhibition means being 0, 2.4, 0 and 6.6%, respectively, LSD=8.8). For the 30 min treatment, there was no significant difference in mycelium inhibition at 45, 47, 50 and 55°C (96.6, 98.2, 96.6 and 100%, respectively).

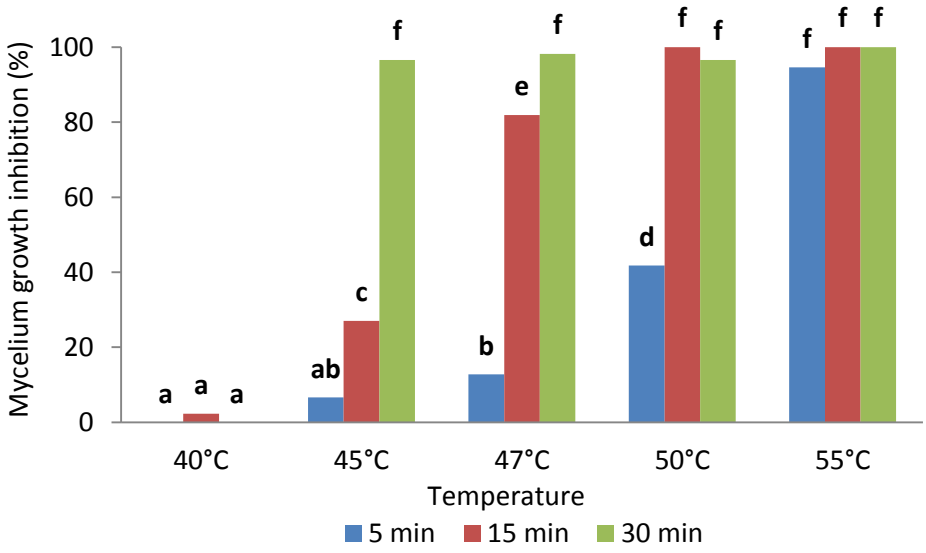


Figure 3.3 Percent mycelium growth inhibition of *Cylindrocarpon* spp. compared to the untreated controls (zero min) after hot water treatment (HWT) at 40, 45, 47, 50 and 55°C for and 5, 15 or 30 min. Results are the mean percent of three replicates for each isolate for each temperature/time combination (P≤0.05, LSD=8.8).

For the 15 min treatments, mycelium inhibition was significantly different between 40, 45 and 47°C (2.3, 27.0 and 81.9% inhibition, respectively) and similar for the higher temperatures of 50 and 55°C with complete inhibition (100%). There was a significant interaction between isolates and HWT temperature (P<0.001, LSD=15.25, Appendix D.1.1) and isolates and HWT time (P<0.027, LSD 11.81, Appendix D.1.1) as detailed in the effects of each temperature below (Figure 3.4) and a significant interaction between isolates, temperature and time (P≤0.05, LSD=26.41), as shown in Figure 3.4. As either HWT temperature or HWT time increased, so did the inhibition of mycelium growth of all isolates. For example, for *C. destructans* isolate 2D, HWT at 45°C caused mycelium growth inhibition which differed significantly between 5, 15 and 30 min (3, 34, and 100%, respectively; LSD=26.41). However at 47°C, mycelium growth of isolate 2D was much more inhibited than at 45°C, being 22% for 5 min and 100% for 15 min and 30 min (LSD=26.41).

HWT 40°C

HWT at the lowest temperature of 40°C for 5, 15 and 30 min was relatively ineffective at inhibiting growth of the nine *Cylindrocarpon* isolates (P>0.05). The isolate most affected was *C. macrodidymum* 1M (Figure 3.4).

HWT 45°C

HWT at 45°C for 5 min was relatively ineffective for all isolates, with the greatest mycelium inhibition for *C. macrodidymum* isolate 3M (13.7%) which was not significantly different to the other eight isolates, with an inhibition range of 0 to 12.9% (P>0.05, LSD=26.41). The 15 min treatment resulted in differences between some isolates (P≤0.05, LSD= 26.41). For *C. macrodidymum* isolate 3M mycelium growth was significantly inhibited compared to all other isolates (73.2%). The 15 min treatment caused significantly greater inhibition for *C. destructan*s isolates 1D, 2D and 3D (44.2, 33.8 and 40.0%, respectively) than the 5 min treatment (6.3, 3.5 and 0%, respectively) and also significantly greater inhibition than isolates of *C. liriodendri*, 3L (5.8%) and *C. macrodidymum*, 1M (0.5%). Mycelium inhibition at 45°C for 5 and 15 min for *C. liriodendri* isolates, 1L, 2L and 3L was not significantly different (13.5, 18.5, and 5.8%, respectively and 12.9, 0 and 2.5%, respectively). When the HWT time was increased to 30 min, mycelium growth was completely inhibited (100%) for all isolates except *C. liriodendri* isolate 3L (69%, LSD=26.41), although mycelium inhibition for two of the

three replicate plates of 3L was 100%, growth of one replicate was inhibited by only 8%, which accounted for the 69% mean.

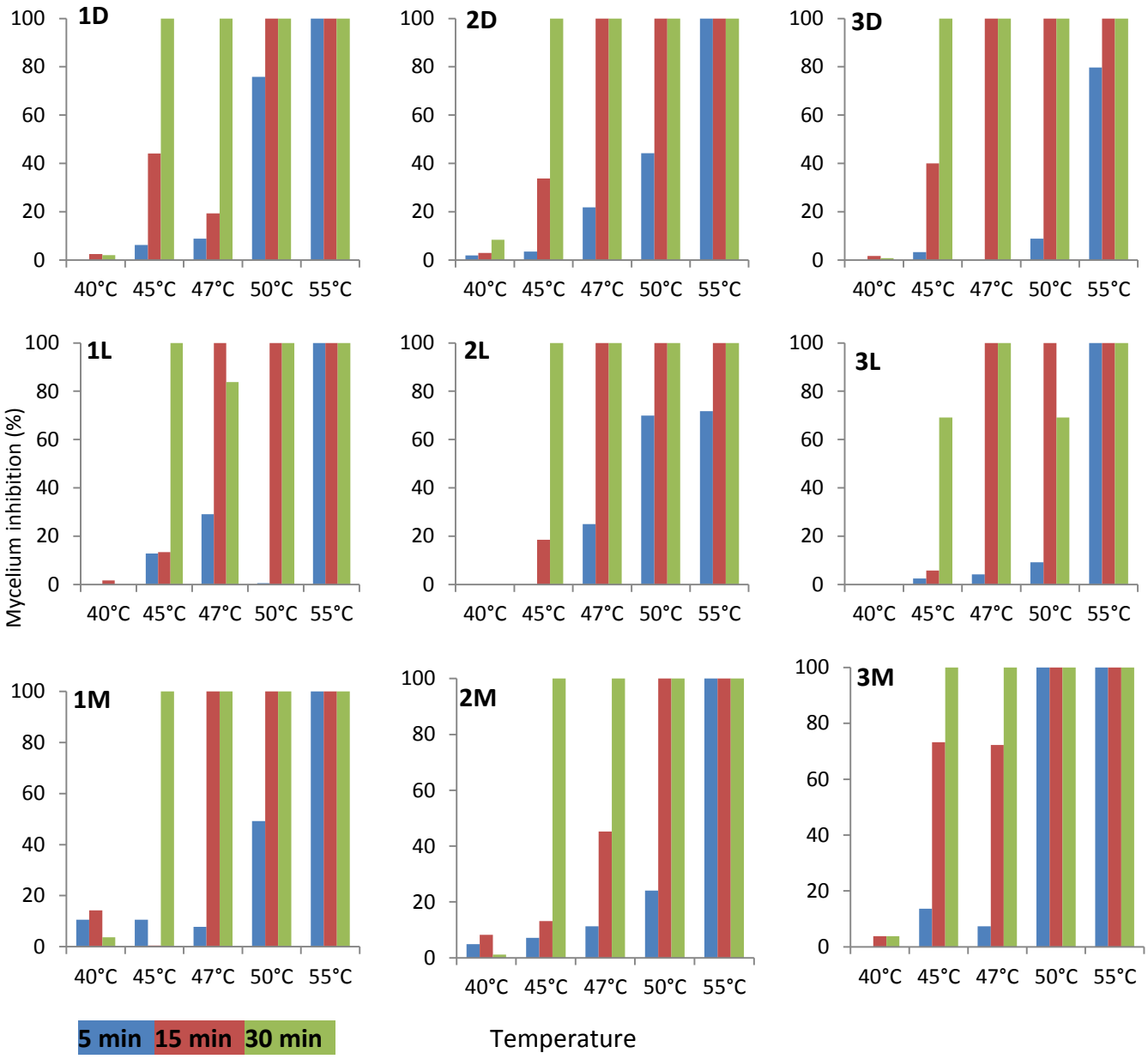


Figure 3.4 Percent mycelium growth inhibition of *Cylindrocarpon* isolates *C. destructans* 1D, 2D and 3D, *C. liriodendri* 1L, 2L and 3L and *C. macrodidymum* 1M, 2M and 3M compared to the untreated controls (zero min) after hot water treatment (HWT) at 40, 45, 47, 50 and 55°C for 5, 15 or 30 min. Results are the mean percent of three replicates for each isolate for each temperature and time combination ($P \leq 0.05$, LSD= 26.41).

HWT 47°C

HWT at 47°C for 5 min was relatively ineffective, with mycelium inhibition ranging from zero for *C. destructans* isolate 3D to 29.2% for *C. liriodendri* isolate 1L, which were significantly different (P≤0.05, LSD=26.41). Mycelium inhibition for the other seven isolates was less than 25% and therefore similar to isolate 3D. The 15 min treatment was more effective and completely inhibited mycelium growth for *C. destructans* isolates 2D and 3D, all three *C. liriodendri* isolates *and C. macrodidymum* isolate 1M. Mycelium growth inhibition of *C. destructans* isolate 1D and *C. macrodidymum* isolates 2M and 3M were significantly different (19.3, 45.2 and 72.2%, respectively LSD=26.41). The 30 min treatment completely inhibited mycelium growth for all isolates except *C. liriodendri* isolate 1L (84%). Although mycelium inhibition for two of the three replicate plates of 1L was 100%, growth of one replicate was inhibited by only 39%, which accounted for the 84% mean.

HWT 50°C

HWT at 50°C for 5 min completely inhibited mycelium growth of *C. macrodidymum* isolate 3M (100%), which was significantly different (P≤0.05, LSD=26.95) to the inhibition for isolates 1M and 2M (49 and 24%, respectively). Mycelium growth inhibition was significantly different between the *C. destructans* isolates 1D, 2D and 3D (75.8, 44.3 and 8.9%, respectively). For *C. liriodendri* isolates 1L and 3L growth was slightly inhibited, with means of 0.6 and 9.1%, respectively and much inhibited for isolate 2L (70%). When the treatment time was increased to 15 and 30 min, mycelium growth was completely inhibited for all isolates except for *C. liriodendri* isolate 3L (69%) and again mycelium inhibition for two replicates was 100% and one replicate was inhibited by only 8% which accounted for the 69% mean.

HWT 55°C

HWT at 55°C for 5 min completely inhibited mycelium growth of seven isolates (100%), to which *C. liriodendr*i isolate 2L (71.8%) was significantly different (P≤0.05, LSD=26.41), but not different to isolate 3D of *C. destructans* (79.7%). For both these isolates two of the three replicate plates showed 100% inhibition. Mycelium growth was completely inhibited for all nine *Cylindrocarpon* isolates at 55°C for 15 and 30 min.

3.4.2 Conidium suspensions

The effect of HWT was significant for *Cylindrocarpon* species (P<0.001, LSD=0.01, Appendix D.1.2), isolates (P<0.001, LSD=0.02, Appendix D.1.2) and all interactions (P<0.001) although the mean differences were very small. These effects were related to only the lowest HWT combination of 40°C for 5 min, since conidium germination was completely inhibited (100%) for all isolates by HWT temperatures or times greater than that. The 24 h continued incubation showed that there was no germ tube development for any of the nine *Cylindrocarpon* isolates, which indicated that conidia were dead after HWT at 40, 45 and 47°C for 15 min.

The means for the species after treatment at 40°C for 5 min showed that inhibition of conidium germination was significantly less for *C. destructans* than *C. liriodendri,* which was significantly less than for *C. macrodidymum* (98.31, 99.06, 99.14%, respectively, LSD=0.01). Conidium germination was significantly different for all isolates (Figure 3.5) except *C. liriodendri* isolates 1L and 3L which were the same (99.06%, LSD=0.02).

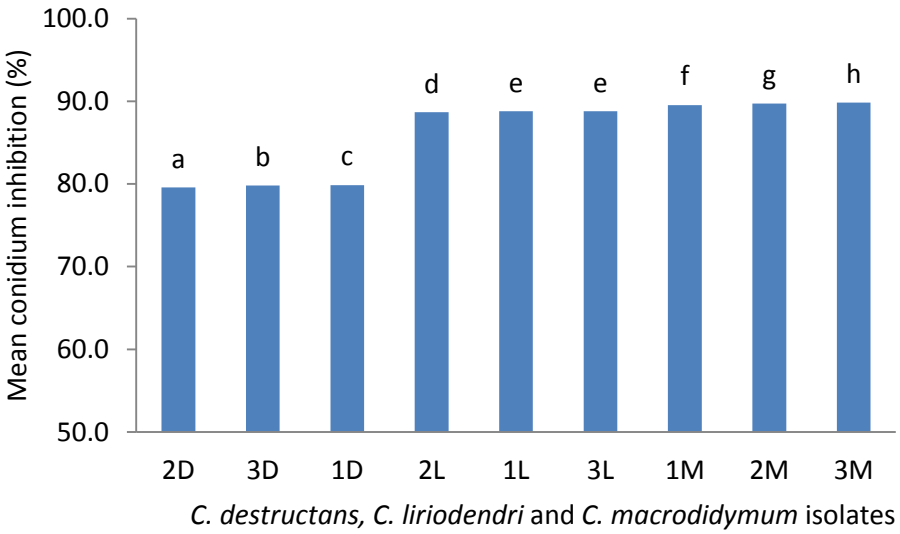


Figure 3.5 Mean percent inhibition of conidium germination compared to the untreated control for nine *Cylindrocarpon* isolates, after HWT at 40°C for 5 min. Bars with the same letter are not significantly different according to Fisher's protected LSD (P≤0.001 LSD=0.02).

Statistical analysis showed that there were significant interactions between all factors tested in this experiment, isolates, HWT temperature, isolates and HWT time (P<0.001, Appendix

D.1.2). However, with only the lowest heat treatment showing less than 100% inhibition and very low LSDs, these were not considered to have biological significance.

3.4.3 Trunk inoculation

The mean percent *Cylindrocarpon* incidences for wood pieces were significantly greater ($P<0.001$, Appendix D.1.4) for those plated from the inoculation site (0 cm) than 1 cm above and 1 cm below that site (47.7, 27.9 and 20.5%, respectively, LSD=9.13) for the HWT trunks. The data were combined and expressed as a percentage of infected wood pieces from each plant (incidence). The effect of HWT was significant for *Cylindrocarpon* species, temperature and interactions between species and temperature ($P<0.001$, Appendix D.1.3), and species x isolate x temperature ($P=0.029$, Appendix D.1.3) but the species x isolate interaction was not significant ($P=0.260$, Appendix D.1.3).

Disease incidence for HWT trunks differed overall between temperatures ($P\leq0.001$, Appendix D.1.4) being 18.5, 8.6 and 1.9% respectively for 47, 48.5 and 50°C, respectively (LSD=8.73). Incidences in HWT treated plants inoculated with *C. macrodidymum, C. destructans* and *C. liriodendri* differed between species (19.1, 5.3 and 4.6 %, respectively, LSD=8.62) and were significantly different to the inoculated controls (73.6, 47.2 and, 76.4%, respectively, LSD=10.69. The interaction between species and HWT temperature showed that disease incidence was significantly greater for *C. macrodidymum* than *C. destructans* and *C. liriodendri* after treatment at 47 and 48.5°C (35.2, 8.3 and 12.0%, respectively, for 47°C and 21.3, 2.8 and 1.9%, respectively, for 48.5°C) but not 50°C when incidences were similar (0.9, 4.6 and 0%, respectively, LSD=15.12).

When the analysis was conducted without the control data, the interaction between species, isolate and temperature was not significant ($P=0.794$), however, when control plant data were included in the analysis for all isolates (Figure 3.6), disease incidence at 47, 48.5 and 50°C was significantly reduced compared to the control of that species ($P=0.029$, LSD=22.68) except for *C. macrodidymum* isolate 1M at 47°C (47.2%) which was similar to the control (66.7%).

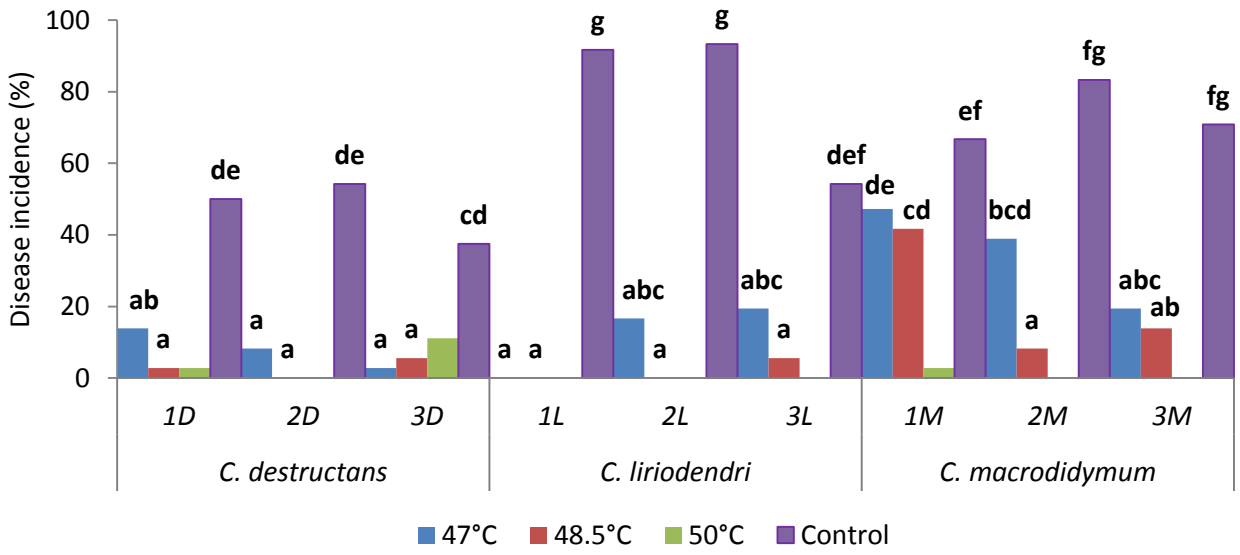


Figure 3.6 Mean pathogen incidence after nine *Cylindrocarpon* isolates grown inside grapevine canes were HWT at 47, 48.5 and 50°C for 30 min. Bars with the same letter are not significantly different according to Fisher's protected LSD (P≤0.001 LSD=22.68).

This interaction showed that some isolates were generally more resistant than others and these differences diminished as the HWT temperature increased. For example, disease incidence for *C. macrodidymum* isolate 1M incidence was 47.2, 41.7 and 2.8%, after treatment at 47, 48.5 and 50°C, respectively, and for isolate 2M, incidence was 38.9, 8.3 and 0.0%, respectively (LSD=22.68).

3.5 Discussion

This study on HWT showed species and isolates variations with respect to mycelium growth and conidium viability using different HWT temperature/time combinations. With increased HWT temperature and time combinations mycelium growth and conidium germination were progressively more inhibited. However the viability of mycelium was less affected by HWT than that of conidia, an overall outcome which concurs with Gramaje *et al.* (2010), who investigated the *in vitro* effects of HWT at 41-55°C for 30-45 min on inhibition of mycelium growth and conidial germination for single isolates of three black foot fungal species and eight *Phaeoacremonium* species.

Gramaje *et al.* (2010) reported 100% mycelium growth inhibition for a single isolate of *C. liriodendri* after 30 min at 48°C and for *C. macrodidymum* after 30 min at 49°C. For the other black foot pathogen, *Cadophora luteo-olivacea* reported once only in New Zealand (Manning and Mundy, 2009), Gramaje *et al.* (2010) reported complete mycelium growth inhibition required 55°C for 30 min and intermediate effects were achieved with lower temperature treatments. For their lowest treatment time of 30 min, they found that 43°C inhibited mycelium growth of the *C. liriodendri* and *C. macrodidymum* single isolates by 75.8 and 74.2%, but complete inhibition of mycelium growth for both isolates required 30 min HWT at 48 and 49°C, respectively, which indicated that the *C. liriodendri* isolate was slightly more sensitive to HWT than the *C. macrodidymum* isolate. The current study showed that mycelium growth of New Zealand *Cylindrocarpon* isolates appeared to be more sensitive to HWT than the Spanish isolates of *C. macrodidymum* and *C. liriodendri* since mycelium growth of the New Zealand isolates was completely inhibited after HWT at slightly lower temperatures and times. Comparisons could not be made regarding *C. destructans* spp. as they were not investigated in the study by Gramaje *et al.* (2010).

HWT of 45°C for 30 min completely inhibited mycelium growth of all three *C. macrodidymum* isolates, the three *C. destructans* isolates and two *C. liriodendri* isolates but not 3L (69% inhibition). The HWT of 47°C for 30 min inhibited mycelium growth for all *Cylindrocarpon* isolates except *C. liriodendri* isolate 1L (84% inhibition). Since two of the three replications for isolate 1L, recorded zero mycelium growth after 47°C treatment, the 84% inhibition of the third replicate was unexpected. A similar result was shown for isolate 3L at 50°C for 30 min, with 69% inhibition. Again, two of the three replicates recorded zero mycelium growth.

Conidial germination in the current study was completely inhibited after very little heat treatment, being 40°C for 15 min or 45°C for 5 min. After 5 min at 40°C, germination rates differed slightly between species, being 79.7, 88.7 and 89.7%, respectively for *C. destructans*, *C. liriodendri* and *C. macrodidymum.* In contrast, Gramaje *et al.* (2010) found that much greater heat treatments were required to provide 100% inhibition of germination. They found that the lowest HWT temperature tested of 41°C for 30 min reduced conidium germination of *C. liriodendri* and *C. macrodidymum* isolates to 38.8% and 65.7% respectively, but that complete inhibition required 30 min treatment at 45°C for *C. liriodendri,* 46°C for *C. macrodidymum* and 51°C for *Ca. luteo-olivacea.* The current research clearly showed that

conidia of the New Zealand *Cylindrocarpon* isolates were more sensitive to HWT than those of the Spanish isolates. The differences in methods were unlikely to be responsible for the differences in results, although the heating of 120 µL of spore suspension in a PCR temperature cycler in this study was likely to be slightly faster than the heating of I mL suspension in a water bath, as used by Gramaje *et al.* (2010). This study showed there were significant differences between isolates from a species with respect to mycelium growth after different HWT treatments. This indicates that many more isolates should be tested from both countries before a final conclusion can be reached about the effects of climate on tolerances to heat treatment, as suggested by Crocker *et al*. (2002).

When the inoculum of the isolates was inserted into canes, and mycelium allowed to grow in each cane sample prior to HWT, 48.5 and 50°C were similarly effective with 50°C treatment reducing incidence to 4.6, 0.0 and 0.9% for *C. destructans, C. liriodendri* and *C. macrodidymum,* respectively. The species most sensitive to heat when growing within wood appeared to be *C. liriodendri* and *C. destructans,* since even 47°C was able to reduce their mean incidences to 12.1 and 8.3%, respectively which was significantly lower than the 35.2% for *C. macrodidymum.* The greater resistance of *C. macrodidymum* at the lower HWT temperatures was not reflected by the effects observed in the *in vitro* experiments, since HWT of 45°C for 30 min completely inhibited mycelium growth of the three *C. macrodidymum* isolates. Isolate 3L was again the least affected of the *C. liriodendri* isolates by treatment of mycelium plugs and in wood, which indicated its greater tolerance to heat. This did not appear to be related to its place of origin since isolate 3L and *C. macrodidymum* 1M were both isolated from grapevines grown in the Hawke's Bay and HWT effects to mycelium growth and conidium germination to these isolates were significantly different which suggests an isolate difference. However more isolates from different regions would need to be tested to support this hypothesis.

The uneven trends observed in inhibition across the HWT temperatures on a few occasions indicated the potential for experimental error. However, they may also have been caused by presence of occasional chlamydospores, which are more heat resistant than conidia and mycelium (Smith *et al.*, 2009). Halleen *et al.* (2004b) reported that *C. liriodendri* and *C. destructans* readily produced chlamydospores when growing on agar, unlike *C. macrodidymum* which rarely produced them. Since chlamydospores may have been present

in some mycelium plugs, this may account for the greater resistance of some replicates, for example one replicate of 3L at 45°C. However, this was unlikely to have caused the increased resistance to HWT observed for *C. macrodidymum* inside the trunk pieces since it produces few chlamydospores in agar. In contrast however this species was reported to produce chlamydospores in soil. Probst (2011) reported that when mycelium and conidia of *C. liriodendri*, *C. destructans* and *C. macrodidymum* were placed into soil, they were all 'converted to chlamydospores or degraded rapidly' although chlamydospores were produced most slowly for *C. macrodidymum*.

A HWT of 55°C for 5 min is typically used as a prophylactic treatment to eradicate exogenous pests and pathogens and while this treatment was very effective at killing conidia it is unlikely to be effective against *Cylindrocarpon* species since the plants are often internally infected by the time they are HWT before sale. While the study by Gramaje *et al.* (2010) suggested that the industry standard HWT of 50°C for 30 min may be sufficient to control *Cylindrocarpon* spp. of Spanish origin, a modified lower HWT may be as effective for New Zealand *Cylindrocarpon* isolates. The cane inoculation experiment showed HWT temperatures at 48.5 and 50°C were similarly and significantly more effective than 47°C for inhibiting mycelium growth. This provides relevant evidence that a lower temperature could be successfully used *in vivo* to rid grapevines of black foot disease but there is little literature to support this hypothesis as these lower temperatures have not been investigated for black foot pathogens.

A similar study to the HWT experiments carried out in this research was able to provide evidence for efficacy of heat treatments lower than the HWT at 57°C for 30 min reported as necessary to control *F. oxysporum* in corms of *Gladiolus* spp. (Tourn.) L (Cohen *et al.*, 1990). More recently, *in vitro* and *in vivo* HWT experiments by Sharma and Tripathi (2008) reported that an *in vitro* study with gladiolus corms artificially inoculated with conidia of *F. oxysporum* f. sp. gladioli and after 30 min subjected to HWT of 45, 50, 55, 60 and 65°C for 5, 15 and 25 min, demonstrated that HWT of 55°C for 25 min was required to kill all conidia. Subsequent *in vivo* experiments treated gladiolus corms 24 h after being inoculated with conidial suspension of the *Fusarium* pathogen and incubated them for 24 h before HWT at 45, 50, 55, 60 or 65°C for 15, 30, 60 or 90 min. The *in vivo* results showed that HWT ≥55°C for 30 min

reduced incidence of fungal infection within corms by 79% compared to controls (Sharma and Tripathi, 2008).

The current study demonstrated that heat treatments lower than the industry standard HWT effectively killed conidia and mycelium of *Cylindrocarpon* spp. and reduced disease incidence in the inoculated canes. However, HWT experiments with naturally infected dormant vines are required since these experiments were not completely representative of commercial HWT with living vines. Even with the inoculated canes, the process of inserting mycelium plugs into the wood and then resealing the hole with compacted sawdust and binding the area with grafting tape may have caused a greater insulation effect, such that HWT had less effect on the pathogen.

3.6 Section 2: - Hot water treatment field experiments

3.7 Materials and Methods

An experiment was conducted in a commercial nursery (Corbans Viticulture, Auckland) to test HWT temperature/time combinations of 47, 48.5 and 50°C for 15 and 30 min since the *in vitro* experiments showed that these HWT significantly reduced pathogen incidence caused by *Cylindrocarpon* spp.

3.7.1 Inoculum

Cultures of the nine *Cylindrocarpon* isolates of *C. destructans* (1D, 2D and 3D), *C. liriodendri* (1L, 2L and 3L) and *C. macrodidymum* (1M, 2M and 3M) were prepared (as described in Section 1 of this chapter) to provide the mixed inoculum of $1x10^4$ conidia/mL to be tested under field conditions.

3.7.2 Rootstock Material

The grape rootstock varieties 101-14 and 5C supplied as dormant cuttings by Corbans Viticulture were prepared as described in Chapter 2.

3.7.3 Field Site Preparation

Preparation of the field site, the planting holes and inoculation of the soil with the mixed conidium suspension of *Cylindrocarpon* isolates was carried out as described in Chapter 2. In brief, 20 mL of the conidium suspension was added to each hole using the drench pack and gun (Figure 2.4b). The rootstock cuttings were planted in double rows of six plants with 100 mm between each plant, therefore there were 12 plants per plot but only the centre eight plants of each plot were assessed for effects of *Cylindrocarpon* spp. infection as described previously (Chapter 2).

3.7.4 Treatment

The experiment was planted in September 2007 and was arranged in a completely randomised split plot (rootstock varieties 101-14 and 5C) design with six blocks, each containing 14 plots 500 mm long, separated by 200 mm buffer zones. The seven treatments were (1) water for the inoculated control (no HWT) and treatments at (2) 47°C for 15 min (3) 47°C for 30 min, (4) 48.5°C for 15 min, (5) 48.5°C for 30 min, (6) 50°C for 15 min and (7) 50°C for 30 min. The bases of the callused rootstock cuttings were re-hydrated in water for 1-3 h before they were planted into the soil which had been infested with the inoculum of the *Cylindrocarpon* spp. immediately prior to planting as described in Chapter 2.

The experimental site was managed by the on-site nursery staff according to standard nursery practices (described in Chapter 2). The plants were grown for 8 months and when they were dormant, were lifted from the nursery using a modified potato lifter and then removed by hand (described in Chapter 2). Immediately after lifting, the plants were washed under running tap water and tied into bundles for each plot and those allocated to treatments 2 to 7 were HWT, using the appropriate temperature/time combination for each treatment, at the Corbans Viticulture HWT facility, with the assistance of their staff.

3.7.5 Hot water treatment facility

The custom designed HWT facility at Corbans Viticulture had three 7,500 L water tanks (Figure 3.7a). The vines were first placed into a wire mesh basket and placed into a tank of cold water for 10-15 min. They were then placed into a fully insulated tank of hot water at the designated temperature for the designated time and immediately after HWT the vines

were placed into the cold water tank for 30 min. The temperature and time were maintained by a computer programme connected to sensor probes permanently fixed inside the tank near the water entry pipe and a portable insertion probe placed into the centre of the vines in the basket (Figure 3.7c). Once the basket had been lowered into the tank (Figure 3.7c) it took approximately one min to get up to required tank temperature (Graham pers comm. 2008), at which time the recording of HWT time began. After HWT and cooling, the treated plants were air-dried for 30 min and packed into perforated plastic bags, placed in cardboard boxes and cool stored (2-4°C) until required.

Annual calibration of the sensor probes (Metermaster New Zealand Ltd) ensured their accuracy to a calibration standard of error of ±0.5°C. The HWT temperature was maintained throughout the duration of the treatment, being managed by the two sensor probes. The temperature was displayed on a computer screen, (Appendix D.2.3) during HWT of 48.5°C for 30 min on which a brown line represented the temperature of the tank sensor probe and the purple line represented the portable sensor probe which was placed into the centre of the vine bundle. These temperatures were maintained at an average temperature of 48.5°C with slight fluctuations in the target temperature during the 30 min treatment (48.47, 48.66, 48.37 and 48.52°C, Appendix D.2.3).

Figure 3.7 Corbans Viticulture hot water treatment (HWT) facility (a) three 7,500 L water tanks, (b) computer monitoring controls that show records of water temperature, (c) bundles of plants lowered into the HWT tank (d) bundles of HWT rootstock plants packed into boxes for cool storage.

3.7.6 Assessment

The winter dormant plants were lifted from the Auckland site in May 2007 and after HWT were immediately transported to Lincoln University for assessment as described in Section 2.8.1. After the roots and shoots were removed, the bare trunks that remained were surface-sterilised. Plants that had no shoots and roots at harvest were classed as 'dead plants' but were still assessed for infection. From each plant, the root crown was removed and discarded. A 1-2 mm section was sliced from across the basal end of the trunk (0 cm) and divided into four pieces which were placed equidistantly around the perimeter of a PDAC plate. Another 1-2 mm slice was cut from further up the trunk (5 cm) to assess the progression of the pathogen, and it was transferred to the centre of the same PDAC plate.

Plates were incubated at 20°C for 7 d and then assessed for presence of *Cylindrocarpon*-like isolates growing from the wood pieces.

3.7.7 Statistical Analysis

The data of *Cylindrocarpon* spp. disease incidence (from 0 cm and 5 cm isolations) and severity (the mean proportion of tissues infection at 0 cm) were analysed by general linear model (GenStat Release 14.1, VSN International Ltd, U.K.) with terms appropriate to the design and the two-way interactions amongst the factors of interest. Where significant main effects or two-way interactions were identified, the significance of differences between individual treatments was further explored using standard errors or Fisher's protected LSD tests. A P-value of ≤0.05 was taken to indicate statistical significance.

3.8 Results

The treatments had significant effects on percent disease severity and incidence (P<0.001, Appendix D.2.1 and D.2.2, respectively) in plants at 0 cm, which were significantly reduced by the HWT treatments (P≤0.05) compared to the inoculated control plants.

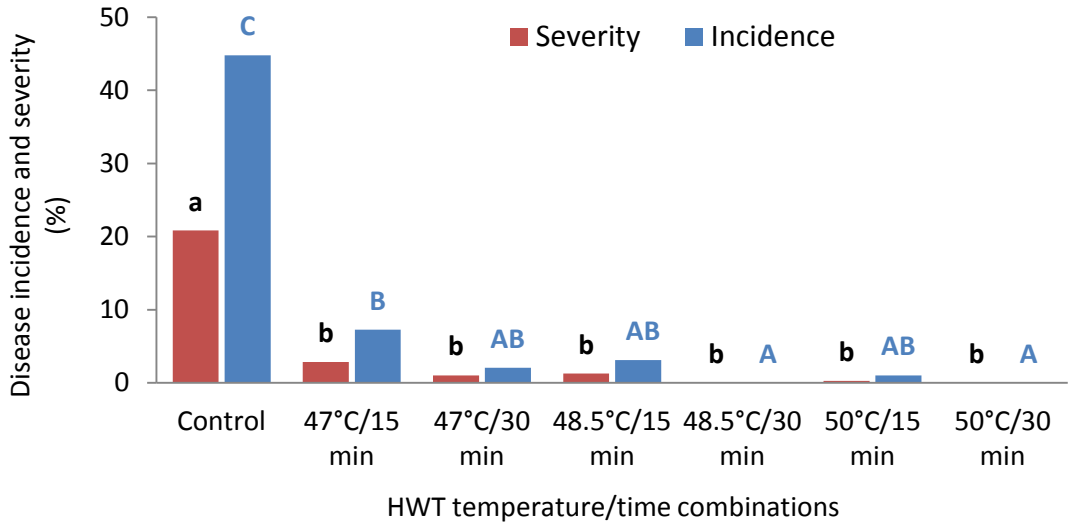


Figure 3.8 Mean percent incidence and severity of *Cylindrocarpon* spp. in grapevine samples after hot water treated (HWT). Columns which are the same colour, blue for disease incidence LSD=7.257 and red for disease severity LSD=4.738, with the same letters above are not significantly different (Fishers protected LSD P<0.05).

The most effective HWTs that resulted in zero disease incidence for plants were 48.5 and 50°C for 30 min (Figure 3.8). However, these zero incidences did not differ significantly from the disease incidences after HWT at 48.5 and 50°C for 15 min and 47°C for 30 min (3.13, 1.04 and 2.08%, respectively LSD=7.26). The greatest disease incidence was in plants that had been HWT at 47°C for 15 min (7.3%), but this was significantly less than disease incidence in control plants (44.8%) as illustrated in Figure 3.8.

All plants that were HWT had similar disease severity which differed significantly from the inoculated control plants ($P \leq 0.05$, LSD=4.74). The trends in disease severity reflected those for disease incidence (Figure 3.8) in that plants that were HWT at 48.5 and 50°C for 30 min had zero disease severity. Disease severity for plants that were HWT at 47, 48.5 and 50°C for 15 min was 2.86, 1.30 and 0.26%, respectively, and was 1.04% for HWT at 47°C for 30 min, which was significantly less than in the control plants (20.83%).

There was no significant difference in disease incidence and severity between rootstock varieties ($P=0.225$) nor any interaction between HWT treatment and rootstock varieties ($P=0.540$) although mean disease incidence and severity were less in rootstock variety 5C (7.14 and 3.42%, respectively) than rootstock variety 101-14 (9.52 and 4.09%, respectively).

3.9 Discussion

This experiment demonstrated the capability of modified HWT temperature/time combinations to eliminate *Cylindrocarpon* spp. from grapevine rootstocks with less heat treatment than the industry standard treatment of 50°C for 30 min. The modified HWT of 48.5°C for 30 min was found to be as effective as the industry standard HWT in eliminating *Cylindrocarpon* spp. from the rootstock plants, which had become naturally infected while growing in soil infested with the black foot pathogens. The modified HWT of 48.5 and 50°C for 15 min did not completely eliminate the pathogens but the levels of disease incidence and disease severity were low. The HWT at 47°C for 15 and 30 min was less effective but still significantly reduced infection by *Cylindrocarpon* spp. compared to control plants.

In both the Auckland and Blenheim experiments described in Chapter 2 (Tables 2.8 and 2.11, respectively), the most effective treatment was HWT of the dormant plants (47°C for 30 min). In the current study, the 47°C temperature for 30 min reduced incidence and severity to 4.6 and 5% respectively, and these results were similar to the results for the Blenheim

experiment in Chapter 2 (5.4 and 4.3%, respectively) but less than the treatment effect in the Auckland experiment (20.6 and 21.9%, respectively). However, the incidence in control plants from Blenheim and Auckland were 58.3 and 70.8%, respectively, which were greater than in this experiment (44.8%) and may have affected the rate of infection elimination from the plants.

In a study carried out in a field nursery at Corbans Viticulture, Dr Anna Graham found that 47 and 48°C for 30 min gave similar results in terms of 'pathogens survival and growth rates' (A. Graham pers comm. 2008) which agreed with the current findings in which infection was similarly reduced with HWT of 47°C and the slightly higher temperature of 48.5°C for 30 min. However in Dr Graham's study when the temperature was increased to 49°C plant viability was marginal for some varieties because the survival and growth rates were lower (data not supplied). In a subsequent experiment (data not supplied) Dr Graham tested HWT durations of 20, 30 and 40 min and found that 20 and 40 min had higher 'reject rates and deaths' than 30 min treatments (A. Graham, pers comm. 2008). As a result of those findings, the HWT protocol at Corbans Viticulture was standardised to a temperature range of 47-48°C (± 0.5°C) for 30 min. Plant viability was not tested in the current study as the plants were not grown on after HWT and highlighted a need for further research in this area.

Grapevine tissues are reported to be less likely to suffer HWT injury if grown in warmer regions than in cool regions (Crocker *et al.*, 2002), most likely because grapevines grown in warmer climates have been reported to have more thermotolerance than those from cooler regions (Crocker and Waite, 2004). This thermotolerance is attributed to the plant's ability to synthesis heat shock proteins (HSP) in the field during hot weather which persist into dormancy and provides protection to the plant during HWT (Crocker and Waite, 2004). Because of the increased tolerance of grapevines to HWT in regions warmer than New Zealand, those research programs have mainly used HWT protocols with temperatures equal to or above 50°C, for example, 50°C in South Africa (Crous *et al.*, 2001; Fourie and Halleen, 2004) and Australia (Edwards *et al.*, 2004; Waite and May, 2005), 51°C in California (Rooney and Gubler, 2001; Whiting *et al.*, 2001) and 50 to 53°C in Spain (Armengol *et al.*, 2007; Gramaje *et al.*, 2009; Gramaje *et al.*, 2010) and Italy (Mannini, 2007). In the latter study, which was conducted over three years, HWT at 52°C for 45 min was found to reduce vine losses after planting to zero to 20% compared to the untreated controls (Mannini, 2007).

Since HWT temperature equal to and above 50°C have not been problematic to the viability of grapevine cuttings grown in warmer climates (Crocker and Waite, 2004; Crocker *et al.*, 2002; Graham, 2007a; Mannini, 2007; Waite and Morton, 2007; Waite *et al.*, 2001), the warm climate grapevine industries have not needed to modify the standard HWT. As a result there was little *in vivo* research done on the effectiveness of HWT temperatures below 50°C for control of grapevine pathogens. However, the *in vivo* experiments in the current research indicate the efficacy of reduced temperatures against New Zealand *Cylindrocarpon* isolates. In addition, much of the research that has been undertaken has focused on controlling Petri disease pathogens of propagation material and not in one year old rootstock plants as treated in the current study. As well, since some results have indicated that the Petri disease pathogens were more tolerant to the industry standard HWT (Armengol *et al.*, 2007; Gramaje *et al.*, 2008; Rooney and Gubler, 2001) than black foot pathogens (Crous *et al.*, 2001; Edwards *et al.*, 2004; Halleen *et al.*, 2007) it is unlikely that HWT temperatures lower than 50°C, as were tested in this study, would be used in warmer climates if the lower temperatures could not control the Petri disease pathogens.

In Spain, *Pa. chlamydospora* and *P. aleophilum* in grapevine cuttings were reported to be less susceptible to the industry standard HWT and required a HWT of 51-53°C for 30 min to eliminate them (Armengol *et al*., 2007). In contrast, in South African field trials conducted over two growing seasons, Halleen *et al.* (2007) reported that HWT at 50°C for 30 min effectively eliminated the Petri disease pathogens, *Pa. chlamydospora and Phaeoacremonium* spp. and the black foot pathogens *C. liriodendri* and *C. macrodidymum* as well as *the Campylocarpon* species, *Campyl. fasciculare* and *Campyl. pseudofasciculare*, from dormant grapevine plants grown in naturally infested soil. The incidence of black foot pathogens in the roots of plants after HWT was zero for both seasons compared to the untreated controls in 2003 (45.3%) and 2004 (16.8%). This agreed with the results of the current research where disease incidence of grapevine samples was zero after HWT at 50°C for 30 min. However, disease incidence was also zero when the temperature was reduced to 48.5°C for 30 min. Halleen *et al*., (2007) also reported that there were zero incidences of *Pa. chlamydospora* or *Phaeoacremonium* spp. compared to the untreated controls in 2003 (21.7% and 6%, respectively) and 2004 (3.5 and 5.3%, respectively). These results showed pathogen, isolate and country variability to HWT and concur with the current research.

In New Zealand, Graham (2007b) reported that vines which had been HWT at 45 and 47°C for 30 min and grown in a field nursery for three weeks had reduced mortality (<10%) compared to vines treated at 50°C, (60%) and reduced 'pathogen' incidence (11 and 3%, respectively) compared to untreated controls (15%). After six months *Pa. chlamydospora* incidence was 5% in vines which had been HWT at 47°C compared to untreated control vines (40%). Although the pathogen incidence was reduced by HWT, the main focus of this research was to improve the number of 'certifiable vines', since earlier experiments by Dr Graham reported the mortality of rootstock cuttings 5C and 101-14 six weeks after HWT at 50°C was 60 and 95%, respectively (Graham, 2007a). This indicated that in a cooler climate, lower HWT temperatures were effective against grapevine pathogens and supported the findings of the current study which showed that HWT of 48.5°C for 30 min reduced *Cylindrocarpon* spp. infection to zero in rootstock plants.

Waite and Morton (2007) reviewed HWT protocols and factors affecting the production of high-quality grapevine planting material and concluded that the cause of physiological damage of cuttings and rooted vines to HWT and various cold storage protocols was not well understood and required further study. Waite *et al.* (2006a) carried out experiments to determine the causes of unexplained plant effects and so investigated the physiological effects of HWT (50°C for 30 min), hydration (8 h post HWT) and cool storage (2-4°C) treatments on grapevine cuttings of Pinot noir and Cabernet Sauvignon. From this study, they were able to hypothesise that losses and delayed growth in HWT cuttings may have resulted from anaerobic cool storage conditions immediately after HWT, which caused post HWT fermentation and by-products of ethanol and acetaldehyde that are toxic to plant tissue (Waite *et al.*, 2006b). They suggested that further investigation by the industry into HWT, hydration and cool storage effects to plants was necessary. They also recommended that nurseries immediately review their HWT and cool storage protocols to use perforated plastic bags to store HWT plant material and that they provide adequate ventilation and air circulation in cool storage facilities to avoid post HWT fermentation and anoxia (Waite *et al.*, 2006a). The results of these experiments will be discussed more fully in the next chapter which investigated the physiological effects of HWT and cold storage on grafted grapevine plants grown in a cool climate.

Chapter 4

Physiological effects of hot water treatment and cold storage on dormant grafted grapevines

4.1 Introduction

The damaging effects of HWT on grapevine cuttings or grafted grapevines, believed by growers to cause bud damage to young grapevines, could be due to physiological changes that may have occurred during the HWT (Waite, 2005). Much of the scepticism that surrounds HWT is based on anecdotal evidence and tends to be centred around *V. vinifera* varieties as opposed to rootstock varieties (Waite *et al.*, 2001). In Australia, the effects of HWT on dormant cuttings of *V. vinifera* cultivars, Cabernet Sauvignon and Pinot noir, have been investigated to (1) develop better nursery protocols (Waite *et al.*, 2006a), (2) examine HWT effects on plant metabolism (Waite *et al.*, 2006b) and (3) examine HWT hydration and cold storage effects on ray cell ultrastructure (Waite *et al.*, 2006c). Those studies indicated that the pre and post handling practices, especially the timing and practices of cold storage of HWT material, may be the underlying reasons for losses of grapevines and not the HWT process itself. Since HWT is one of the few sustainable methods available to nurseries to control exogenous and endogenous pests and diseases of grapevines (Waite and May, 2005), further investigation into HWT effects to young grapevines is required.

A physiological factor that is known to be affected by cold storage is stored starch concentration. Starch is the main compound for carbohydrate (CHO) reserves of grapevines (Winkler and Williams, 1945) and is predominately stored in the root system of vines (Loescher *et al.*, 1990; Winkler and Williams, 1945). Much of the literature on grapevine CHO storage focuses on CHO reserves in canes, cordons and trunks and although the roots of grapevines are a primary source of CHO reserves, little is known of what influences root CHO reserves (Bennett, 2002) in young vines. The current research investigated the effects of harvest month, HWT and cold storage of dormant grapevine plants on concentrations and types of CHOs stored in grapevine root samples, as well as on grapevine growth, and the effect of these treatments on black foot disease incidence and severity.

4.2 Materials and methods

4.2.1 *Cylindrocarpon* isolates

The nine *Cylindrocarpon* isolates of *C. destructans* (1D, 2D and 3D), *C. liriodendri* (1L, 2L and 3L) and *C. macrodidymum* (1M, 2M and 3M) were maintained on SNA slants at 4°C as described in Chapter 2. When required, the isolates were subcultured to PDA plates and incubated at 20°C for 2-4 weeks when mixed isolate conidium suspensions were prepared as described in Chapter 2. These suspensions were put into 2 L bottles and packed in ice within polystyrene containers for transportation to the Auckland field site where they were mixed with enough water to give $1x10^6$ conidia/mL just before soil inoculation the following day.

4.2.2 Grafted grapevine material

Grafted plants of rootstock 101-14 with scion cultivar (cv.) Sauvignon blanc, rootstock 101-14 with cv. Pinot noir and rootstock 5C with cv. Sauvignon blanc were prepared and supplied by Corbans Viticulture in accordance with standard nursery practices. They were cool stored at 2-4°C until required, and then were placed outside in a covered area to acclimatise for one day before planting.

4.2.3 Field site preparation

Preparation of the field site, the planting holes and inoculation of the soil with the mixed conidium suspension of *Cylindrocarpon* isolates was carried out at a Corbans Viticulture nursery site, as described in Chapter 2.

Treatment:	HWT			No HWT			HWT+1 mth cold store			No HWT+1 mth cold store		
X X	X X	X X	X X	X X	X X	X X	X X	X X	X X	X X	X X	X X
X X	X X	X X	X X	X X	X X	X X	X X	X X	X X	X X	X X	X X
Variety:												
101-14 Pinot noir	101-14 Sav. blanc	5C Sav. blanc	5C Sav blanc	101-14 Pinot noir	101-14 Sav. blanc	101-14 Pinot noir	101-14 Sav. blanc	5C Sav. blanc	101-14 Sav. blanc	5C Sav. blanc	101-14 Pinot noir
Block 1 (May)												

Figure 4.1 Experimental design for one of the six blocks and the May harvest treatment, showing the four treatments which were applied to three different grafted grapevine varieties.

The grafted plants were planted in double rows of plants with 100 mm between plants, four plants per plot, which were separated by 200 mm empty buffer zones (Figure 4.1).

4.2.4 Treatments

The field experiment planted in September 2007 contained six replicate blocks, each with 12 plots of four plants with a 500 mm buffer between plots. Plants within each block were arranged in a completely randomised split plot design (three rootstock by scion combinations; Figure 4.1), with four treatments: (1) HWT: 48.5°C for 30 min, (2) no HWT, (3) HWT: 48.5°C for 30 min after 1 month cold storage and (4) no HWT after 1 month cold storage (Table 4.1). To also assess the effects of different harvesting times (May, June and July), which represented different stages of dormancy, the experimental design was replicated at these three times, each in a different area across the same site.

Table 4.1 Treatments carried out on plants harvested in May, June and July before being assessed.

Harvest Month	Treatment	Cold storage	Hot water treatment	Begin assessment
May	1	-	+	May
	2	-	-	May
	3	+	+	June
	4	+	-	June
June	1	-	+	June
	2	-	-	June
	3	+	+	July
	4	+	-	July
July	1	-	+	July
	2	-	-	July
	3	+	+	August
	4	+	-	August

Prior to planting in the field, the bases of the grafted rootlings had been re-hydrated in water for 1-3 h after which they were planted into the infested soil as described in Chapter 2. The plants were managed by the on-site nursery staff according to standard nursery practices (described in Chapter 2).

The plants were harvested in May, June and July the following year (Table 4.1). The dormant, grafted grapevines were trimmed to two nodes in the field immediately prior to lifting (as described in Chapter 2), in preparation for replanting into the greenhouse at Lincoln University. The plants were tied into bundles by plot (four plants) and taken to the HWT facility where all plants were cleaned by holding under running tap water and the roots trimmed to approximately 10-15 cm following standard nursery practices. After cleaning, the plants allocated to Treatment 1 (Trt 1) were HWT at 48.5°C for 30 min at the Corbans Viticulture HWT facility with the assistance of their staff as described in Chapter 3. The plants of Trt 1 and Trt 2 were packed into cardboard boxes and air-transported that afternoon to Lincoln University. The plants of Trt 3 and Trt 4 were packed into cardboard boxes lined with perforated plastic bags, which had ~1 cm holes punched at ~15 cm intervals to allow aeration, and were stored at 2-4°C in the cold storage facility at Corbans Viticulture. After four weeks the plants were removed from cold storage and the Trt3 plants were HWT before air freight of Trt 3 and Trt 4 plants to Lincoln University.

As soon as these plants arrived at Lincoln University, one representative root sample, approximately 10 cm long and 3-5 mm in diameter (Figure 4.3a), was taken from each plant. The four root samples from the four plants in the experimental plot were placed together into a brown paper envelope and oven-dried for 2-3 d at 60°C. They were then stored in airtight containers at 8°C until all samples had been collected and dried for all treatments and months, when they were assessed for CHO content.

The grafted grapevines were then planted into separate 2.5 L plastic pots (Figure 4.2a) containing potting media of 80% bark and 20% pumice (Southern Horticultural Products Ltd South, Christchurch) amended with 2 g/L Osmocote Exact® plus Hydraflo® 1 g/L (Everris, New Zealand) and agricultural lime 1 g/L (Ravensdown New Zealand Ltd). Immediately after potting, the plants were placed onto corrugated steel sheets raised 10 cm above the greenhouse floor. The plants were arranged in as randomised a design as possible (Figure 4.2b) but to avoid contamination by *Cylindrocarpon* spp. the HWT plants were placed onto separate corrugated sheets from the untreated plants and separated by 200 mm. In addition, the plants which belonged to Trt 3 and Trt 4 were placed in the greenhouse alongside the Trt 1 and Trt 2 plants one month later (when returned from cold storage and/or HWT). These grapevine plants were grown in a heated greenhouse (May to

November) and were illuminated by high pressure sodium lamps (Son-T Agro 400, Philips) which were turned on from 4 am to 12 pm and 4 pm to 8 pm to ensure the plants received adequate light for 16 h per day. Temperatures varied from 15 to 30°C and the plants were watered daily, or as needed, by nursery staff.

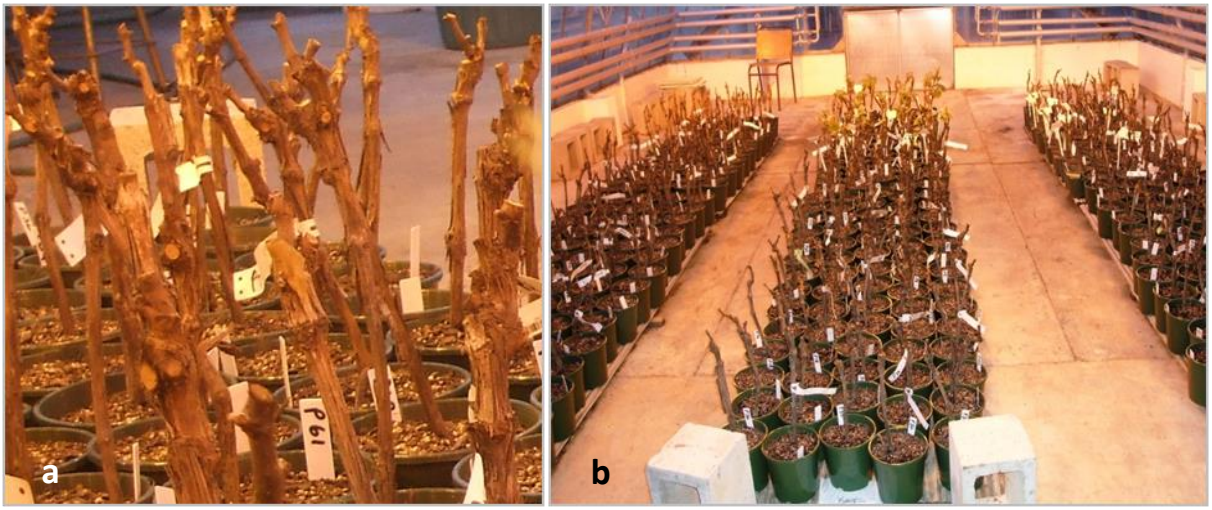


Figure 4.2 Grafted grapevines that were pruned to two nodes and treated before potting up (a). Grafted grapevines in the greenhouse at different stages of bud development (b).

The second harvest of the grafted grapevines was carried out in June; 4 weeks after the May harvest and the third in July, 4 weeks after the June harvest. The processes were the same as for the May harvest, with the last group of plants from July Trt 3 and Trt 4 being transported to Lincoln University for assessment in August 2008 (Table 4.1).

4.2.5 Assessment of bud development

To measure the effect of HWT and cold storage on bud growth, the phenological stage of bud development for each plant was assessed each week using the modified system of Eichhol and Lorenz (E-L), as developed by Coombe (1995) (Appendix E.7.2). The growth stages were recorded as: 1 winter bud or dormancy, 2 bud swell, 3 woolly bud with brown wool visible, 4 budburst, 5 green leaf tips visible, 7 first leaf separated from shoot tip, and 9 two to three leaves separated with shoots 2-4 cm long. The time taken in weeks for each plant to reach growth stage ‘E-L 4’ and ‘E-L 9’ was used as a measure of the HWT and cold storage effects on bud development of the grafted grapevines. After all plants from a

harvest month had reached ‘E-L 9’ they were removed from the greenhouse and assessed for *Cylindrocarpon* spp. infection.

4.2.6 Assessment of *Cylindrocarpon* spp. infection

Plants were lifted out of their pots and the loose potting mix washed from the roots which were then cut from the stem base and discarded. After the roots and shoots were removed, the bare trunks were surface-sterilised as described in Section 2.1.13. From each plant, the root crown was removed and discarded. A 1-2 mm section was sliced from across the basal end of the trunk (0 cm) and divided into four pieces which were placed equidistantly around the perimeter of a PDAC plate. Another 1-2 mm slice was cut from further up the trunk (5 cm) to assess the progression of the pathogen, and it was transferred to the centre of the same PDAC plate. The plates were incubated for 7 d before *Cylindrocarpon* spp. incidence and severity were assessed for each plant as in Section 2.1.13. Data were analysed as described in Section 2.1.14.

4.2.7 Soluble sugar and total starch analyses

The dried root samples per envelope (Figure 4.3a) were each finely ground to a powder (Figure 4.3b) using an ultra-centrifugal mill (RETSCH Ultra Centrifugal Mill ZM 200, Germany) (Figure 4.4a) with a 0.2 mm sieve (Figure 4.4b). The ground root sample for each four plant plot was stored in an airtight container at 4°C until required for CHO assessment.

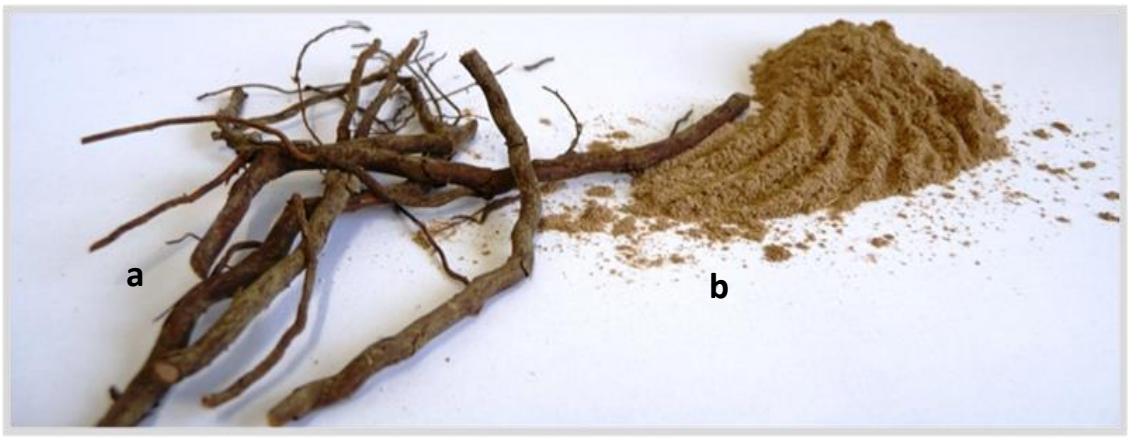


Figure 4.3 Grapevine roots samples (a) and finely ground root samples (b).

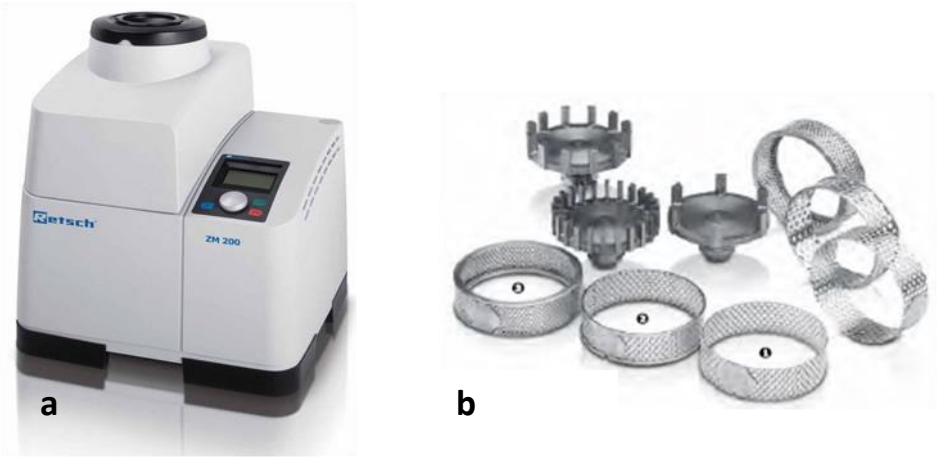


Figure 4.4 The RETSCH Centrifugal grinding mill (a) for processing root samples by impact and shearing between rotor and fixed ring sieve (b) (Retsch, 2006).

4.2.8 Carbohydrate extraction from grapevine roots

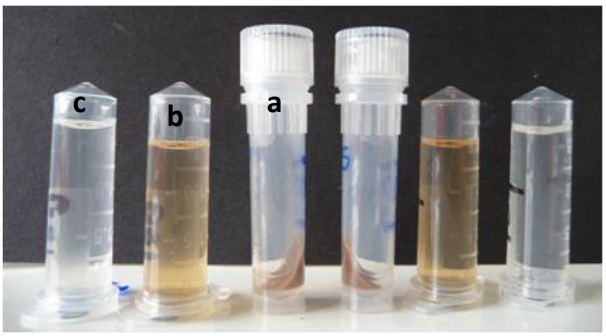
Two standard colorimetric tests were used to measure the concentrations of soluble sugar and starch in the ground grapevine root samples. An anthrone method based on Pollock and Jones (1979) and Jermyn (1956) was used to test for soluble sugars and the Megazyme Assay Kit, which in comprised; the K-TSTA 04/2009 amyloglucosidase/α-amylase method (Megazyme International, Ireland) (McCleary B *et al.*, 1997a, 1997b) was used to determine total starch. However, for both methods the quantities were modified for micro-analysis (Appendix E.8).

4.2.9 Water soluble carbohydrate extraction

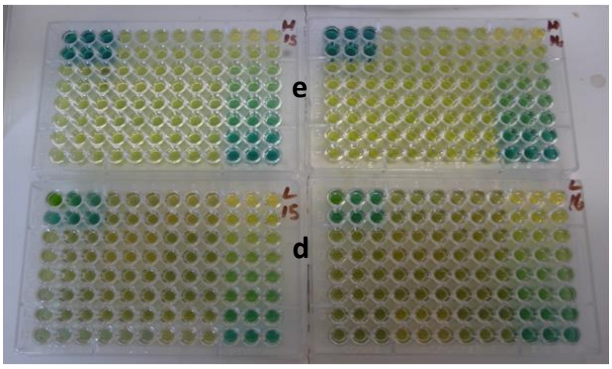
To extract the low molecular weight (LMW) water soluble CHO (WSC) (described in Appendix E.8, Sugar analysis), duplicate samples of 25 mg was used from each of the dried finely ground roots from each plot. Each sub-sample was placed into a 2 mL screw cap tube (Figure 4.5C, a) with 1 mL of 80% ethanol and shaken for 30 min at 65°C in a Labnet® heating block and shaker, (Montreal-Biotech Inc., Canada; Figure 4.5A) and then centrifuged at 21,162 *g* for 15 min (Sorvalls ® Centrifuge, Thermo Fisher Scientific, USA, Figure 4.5B). The supernatant was pipetted off without removing the root sample and the procedure was repeated. The two supernatants from each tube were then combined (Figure 4.5C, b and c).

A. Labnet® heating block and shaker, Montreal-Biotech, Inc. Canada

B. Sorvalls ® Centrifuge, Thermo Fisher Scientific, USA



C. Centrifuged sample (a), supernatants from low (c) and high (b) molecular weight CHOs.



D. Microplates with LMW (d) and HMW (e) supernatant after Anthrone reaction

Figure 4.5 A and B: Equipment used for carbohydrate (CHO) extraction. C: Extracted root pellet (a), low (b) and high (c) molecular weight samples for water soluble CHO measurement of grapevine roots and D: low (d) and high (e) microwell plates containing water soluble CHO samples after colorimetric analysis.

To extract the high molecular weight (HMW) WSC, 1 mL of deionised water was added to the residue pellet from the LMW extraction and shaken for 30 min at 65°C, then centrifuged for 15 min at 21,162 *g*. The supernatant was pipetted off without removing the root sample and the procedure was repeated before the two supernatants were combined (Figure 4.5C, c).

4.2.10 Water soluble carbohydrate analysis

Duplicate WSC samples from LMW extractions (12 µL) were diluted with nanopure water (188 µL) in a 2 mL screw cap tube from which three 40 µL aliquots of WSC extract were pipetted into triplicate wells of a FLUOstar Omega 96 well microplate (InVitro Technologies, Australia). Eight sucrose standards in the range 0 to 100 µL/mL (Appendix E.8) and two pasture control samples (Control (CNT) East, Agricultural and Life Sciences Faculty, Lincoln University) were also pipetted into triplicate wells. To each well was added 200 µL of

anthrone reagent by a FLUOstar Omega UV/Vis absorbance spectrometer (Appendix E.8), and the plate was automatically shaken to mix (in the same machine).

Duplicate WSC samples from HMW extractions (40 µL) were diluted with nanopure water (160 µL) in a 2 mL screw cap tube from which three 40 µL aliquots of WSC extract were pipetted into triplicate wells of a FLUOstar Omega 96 microplate (Figure 4.5e). Eight inulin standards in the range of 0 to 100 µL/mL (Appendix E.8) were also pipetted into triplicate wells. Anthrone reagent (200 µL) was added to each well and mixed as above.

The microwell plates were covered with microwell lids and incubated for 25 min at 65°C, which was sufficient to allow colour reaction, and the absorbency was immediately measured at 620 nm using a FLUOstar Omega UV/Vis absorbance spectrometer (BMG LABTECH GmbH, Germany). Standard curves were derived for the sucrose and inulin standard concentration ranges using the linear regression equation: Y = m x + b (example illustrated Figure 4.6).

Where: Y = absorbance units at 620 nm
m = slope/1000 µg/mL
x = µg sucrose or inulin/mL
b = y-intercept

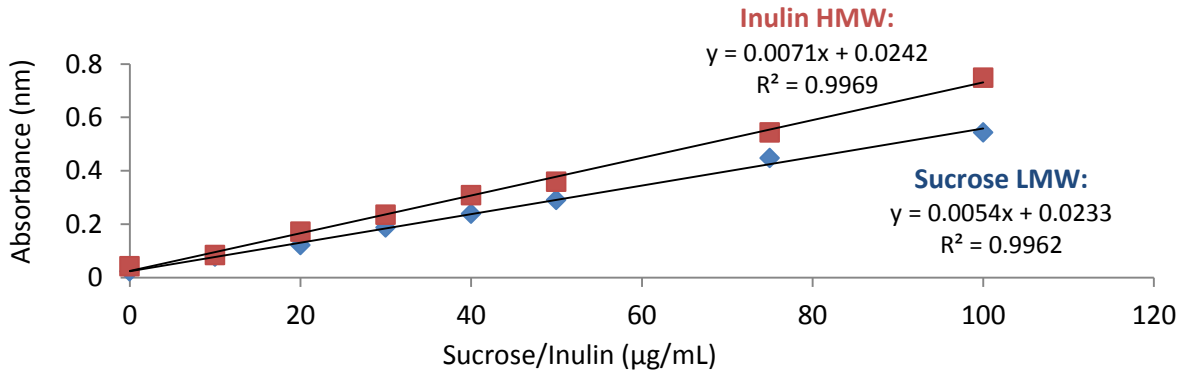


Figure 4.6 Example of anthrone reagent sucrose and inulin standard curve.

The LMW and HMW were combined to give total % WSC sugar per sample using the equation:

HMW WSC = $\underline{(\Delta E - 0.0242)/0.0071\ \mu g/mL}$ = HMW µL total

Dilution factor (DF) = HMW µg total/sample mg x 100 = % HMW

% HMW + % LMW = Total % WSC (Appendix E.10)

Where:

ΔE = the mean absorbance of the samples - absorbance of sample blank/m (620 nm).

4.2.11 Total starch extraction and analysis

The root pellet remaining after soluble sugar extraction was used to determine total starch by enzymatic digestion using the Megazyme Assay Procedure kit K-TSTA 04/2009 which was modified for micro-analysis (described in Appendix E.8 Starch Analysis). The pellet samples were resuspended by addition of 0.3 mL of the dimethyl sulfoxide reagent, capped and vortexed (Model 250VM, Hwashin Technology, Korea) for 15 s at medium speed. The samples were heated in a boiling water bath for 5 min and swirled after 2 and 4 min to improve solubilisation of resistant starch for hydrolysis by amylase enzymes. After removal from the water bath, 0.65 mL (195 U) of thermostable α-amylase (Megazyme, Bottle 1) in MOPS buffer (50 mM, pH 7.0, Appendix E.8) (Megazyme, 2009) was added to the tube and the samples were vortexed for 15 s at medium speed. The tubes were then incubated in a boiling water bath for 6 min and swirled after 2 and 4 min. Samples were cooled by placing them into a 50°C water bath for 20 min and 0.7 mL sodium acetate buffer (200 mM, pH 4.5) was added, immediately followed by addition of amyloglucosidase (20 µL, 0.5 U) (Megazyme, Bottle 2). They were then vortexed as before and the tubes returned to the 50°C water bath for 30 min incubation. After incubation, the samples were centrifuged for 15 min (21,162 *g*) and the supernatant withdrawn for colorimetric analysis.

From the supernatant, duplicate starch samples (9 uL) were pipetted into triplicate wells of a FLUOstar Omega 96 microplate. Five D-Glucose standards (Megazyme, Bottle 5) in the range of 0 to 1000 uL/mL, and two maize control samples, diluted 1:900, (Megazyme, Bottle 6) were also pipetted into triplicate wells. To each well, 270 µL of GOPOD reagent from Megazyme bottles 3 and 4, prepared according to manufacturer's instructions; (Megazyme, 2009) was injected by a FLUOstar Omega UV/Vis absorbance spectrometer and

automatically shaken to mix (in the same machine). The microwell plates were removed, covered with microwell lids and incubated for 30 min at 50°C to allow colour reaction. The absorbency was immediately measured at 510 nm using the same FLUOstar Omega UV/Vis absorbance spectrometer and the standard curve for glucose standards was derived using the linear regression equation: Y = m x + b (example illustrated Figure 4.7):

Where: Y = absorbance units at 510 nm
m = slope/1000
x = µg glucose/mL
b = y-intercept

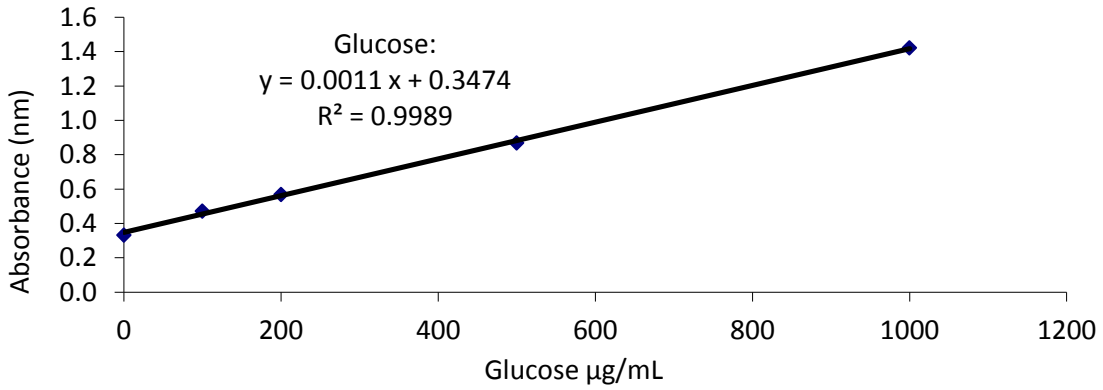


Figure 4.7 Example of anthrone reagent glucose standard curve.

The weight of starch in each sample was calculated based on the Megazyme Starch Assay Kit equation (McCleary B *et al.*, 1997a, 1997b; Rose *et al.*, 1991) and adjusted for micro-analysis. Using the standard curve example in Figure 4.7, the equation was:

= (ΔE - 0.3474)/0.0011 µg/mL

= µg/mL x mL (mL= total extraction volume, 1.67 mL)

(µg total/1000)/sample weight x hydrolysis factor

= mg /mg starch x 100 = % starch

Where:

ΔE = the mean absorbance of the samples - absorbance of sample blank/m (510 nm)

Starch hydrolysis factor 162/180 (= 0.9)

The weight (mg) of starch (example, Appendix E.9) in each sample was converted to a % dry weight (Dwt) using the following equation:

% dry weight = mg starch/sample x 100/sample weight.

4.2.12 Statistical Analysis

The data available for analysis were the *Cylindrocarpon* spp. incidence (0 cm and/or 5 cm) and severity determined from proportion of infected pieces (0 cm). The bud growth data analysed were the times (weeks) taken for each plant to reach 'E-L 9' and 'E-L 4', the mean being determined from the four grapevine plants per plot. The data analysed for CHO analysis were the percentages of total starch and sugar for each plant, the mean determined from the four grapevine plants per plot. Analysis was carried out using a mixed model analysis of variance (ANOVA) with different error terms used for the nested factors, as appropriate to the design (C. Frampton pers. comm., 2012). Where significant main effects or interactions were identified, the significance of differences between individual treatments or treatment combinations was further explored using Fisher's protected LSD tests. Due to the complexity and uniqueness of the design, LSD values were not calculated for those treatments with only two data levels however the differences between the two levels were apparent as shown with the HWT and cold storage results (C. Frampton pers. comm., 2012). A P-value of ≤0.05 was taken to indicate statistical significance.

4.3 Results

4.3.1 *Cylindrocarpon* spp. infection

The month and HWT had significant effects on percent disease incidence and disease severity (P≤0.010 and P≤0.001, respectively, Appendix E.1.1 and E.2.1, respectively), which were significantly reduced (P≤0.05) compared to levels in control plants. Cold storage caused no significant effect (P>0.05) on disease incidence at 0 and 5 cm and disease severity (P=0.40, 0.83 and 0.63, respectively). Significant effects are illustrated in Table 4.2.

Plants that were harvested in May had significantly greater disease incidence (P≤0.05 LSD=9.85) and severity (P≤0.05 LSD=5.70) than plants that were harvested in July (23.6 and 13.5%, and 14.2 and 7.2%, respectively). These monthly differences were significant for non HWT plants but not for HWT plants (Figure 4.8). Plants that were HWT had significantly less (P≤0.05) disease incidence at 0 cm (LSD=9.59) and 5 cm (LSD=7.46), and disease severity (LSD=5.75) (5.6, 1.8 and 3.2%, respectively) than plants that were not HWT (32.4, 15.3 and 19.3%, respectively).

Table 4.2 Significance of month, treatment and variety effect (+ or - meaning P≤0.05 or P>0.05, respectively) for grafted grapevines of two rootstock and two scion varieties that were harvested in May, June and July from a field nursery. They were then treated by HWT and/or cold storage, compared to control plants that were not HWT nor cold stored.

	Significance level P<0.05 (+)					
Treatment effect	**WSC Sugar**	**WSC Starch**	**Bud growth**	**Disease incidence**	**Incidence 5 cm**	**Disease severity**
MONTH	+	+	+	+	-	+
HWT	+	+	+	+	+	+
COLD STORED	+	+	+	-	-	-
HWT *COLD STORED	+	-	+	-	-	-
MTH * HWT	-	-	+	+	-	+
MTH *COLD STORED	+	-	+	-	-	-
MTH *HWT *COLD STORED	+	-	+	-	-	-
VARIETY	+	+	+	+	+	+
MTH * VARIETY	-	-	-	-	-	-
HWT*VARIETY	-	-	-	-	+	+
COLD STORED * VARIETY	-	-	-	-	-	-
HWT*COLD STORED * VARIETY	-	-	-	-	-	-
MTH *HWT *COLD STORED*VARIETY	-	-	-	-	-	-
MTH *COLD STORED*VARIETY	-	-	-	-	-	-
MTH *HWT *VARIETY	-	-	-	-	-	-

There was a significant interaction between harvest month and HWT (Figure 4.8) for disease incidence at 0 cm (P=0.027, LSD=16.6) and disease severity (P=0.037, LSD=10.0).

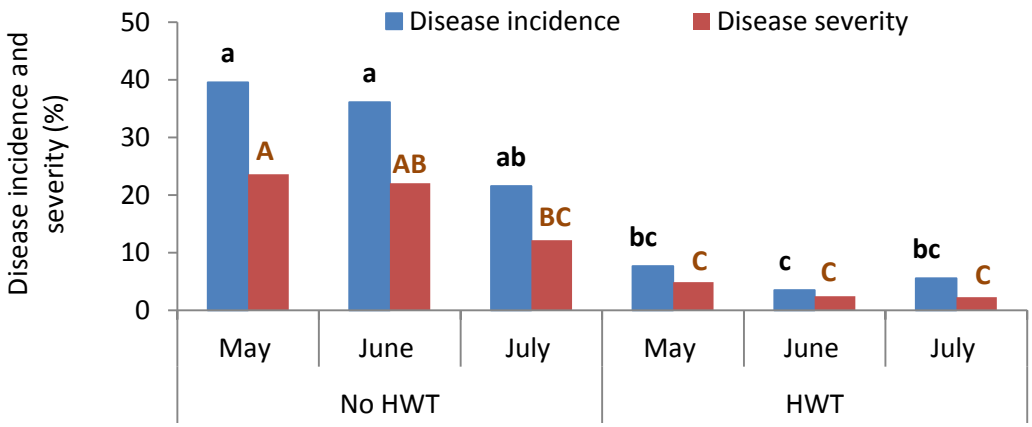


Figure 4.8 Mean percent incidence and severity of *Cylindrocarpon* spp. in grapevine plants that were harvested in May, June and July, with the effects of a hot water treatment (HWT). Columns which are the same colour, blue for disease incidence LSD=16.62 and red for disease severity LSD=10.0, with the same letters above are not significantly different (Fishers protected LSD P<0.05).

These interactions were mainly associated with non-HWT plants having decreasing incidence and severity over the harvest months while HWT plants had similar incidence and severity over the harvest months.

There was a significant variety effect (Figure 4.9) as grafted grapevines of 101-14 cv. Pinot noir had significantly (P≤0.05) greater disease incidence at 0 cm and 5 cm, and disease severity than those of 101-14 cv. Sauvignon blanc and 5C cv. Sauvignon blanc, which were similar (Appendix E.1.1, E.2.1 and E.3.1, respectively).

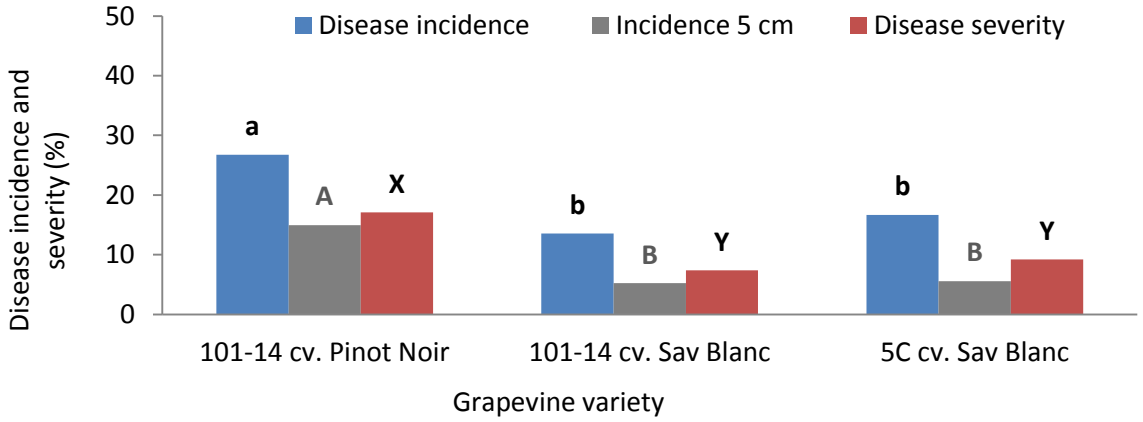


Figure 4.9 Mean percent incidence and severity of *Cylindrocarpon* spp. in grapevine plants of variety 101-14 cv. Pinot noir, 101.14 cv. Sauvignon blanc and 5C cv. Sauvignon blanc. Columns which are the same colour, blue for disease incidence at 0 cm (LSD=6.68) and grey at 5 cm (LSD=4.72) and red for disease severity (LSD=4.38), that have the same letters above are not significantly different (Fishers protected LSD P<0.05).

There was a significant interaction between grapevine variety and HWT primarily due to significantly greater disease incidence at 5 cm (P=0.002) and disease severity (P=0.018) for non-HWT plants of variety 101-14 cv. Pinot noir (26.4%, LSD=6.67 and 28.6%, LSD=6.2, respectively) than varieties 101-14 cv. Sauvignon blanc (8.3 and 12.7%, respectively) and 5C cv. Sauvignon blanc (11.1 and 16.5%, respectively).

4.3.2 Water soluble carbohydrates and bud development

The month (Figure 4.10), HWT, cold storage and variety had significant effects on percent sugar (P=0.023, P≤0.001, P≤0.001, P≤0.001, respectively), percent starch (P≤0.001, P=0.05, P≤0.001, and P≤0.001, respectively) and bud growth to E-L 4 and E-L 9 (all P≤0.001, Figure

4.11). There were also significant interaction effects amongst these factors with respect to percent sugar, percent starch and bud growth to E-L 4 and E-L 9. (Appendix E.5.1, E.6.1 and E.7.1, respectively).

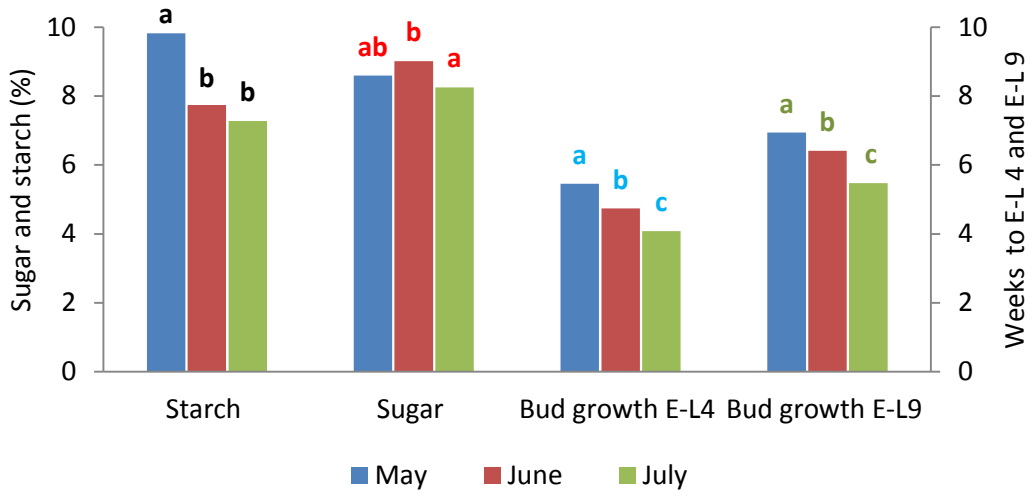


Figure 4.10 Mean percent sugar and starch in grapevine roots and weeks for buds to reach growth stage E-L 4 and E-L 9 for plants harvested in May, June and July after hot water treatment (HWT). Columns for starch (LSD=1.142), sugar (LSD=0.837) and bud growth to E-L 4 (LSD=0.242) and E-L 9 (LSD=0.444) with the same letters above are not significantly different (Fishers protected LSD P<0.05).

4.3.2.1 Effects on bud development

The most rapid growth occurred for plants that were harvested later, with plants from July reaching growth stage E-L 4 significantly faster than those from May and June (4.1, 5.5 and 4.7 weeks, respectively) (Figure 4.10) and this was similar for plants to reach growth stage E-L 9 (5.5, 6.9 and 6.4 weeks, respectively).

There was a significant effect of HWT on growth (P<0.001), with times taken for HWT and non HWT being 4.4 and 5.1 weeks, respectively to E-L 4 and 5.9 and 6.7 weeks, respectively to E-L 9 (Figure 4.11).

Cold storage caused plants to grow more quickly than with non-cold-stored plants (P<0.001) reaching E-L 4 in 4.2 and 5.3 weeks, respectively) and E-L 9 in 5.6 and 6.9 weeks, respectively) (Figure 4.11).

Variety effects were also shown for time taken for plants to reach EL-4 and EL-9, (both P<0.001) with 101-14 cv. Pinot noir fastest at 4.5 and 6.0 weeks, 101-14 cv. Sauvignon blanc 4.7 and 6.2 weeks and 5C Sauvignon blanc 4.9 and 6.5 weeks, respectively, (Figure 4.11).

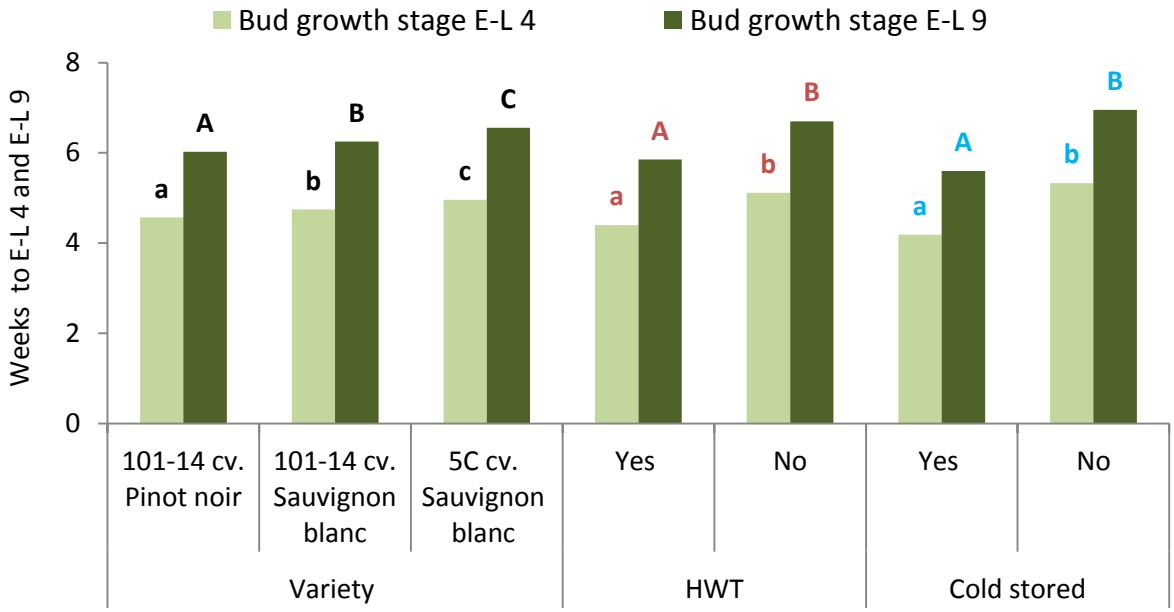


Figure 4.11 Mean hot water treatment effects on bud growth to E-L 4 and E-L 9 for plant varieties (LSD=0.123 and 0.172, respectively), HWT (LSD=0.170 and 0.297, respectively), and cold storage of grapevines (LSD=0.170 and 0.297, respectively). Columns for variety, HWT and cold stored with the same letters above are not significantly different (Fishers protected LSD P<0.05).

The month x HWT interaction (P≤0.001) was evident in that plants harvested in June and May, after HWT reached E-L 4 faster than non-HWT plants (4.3 and 4.8 and 5.1 and 6.1 weeks, respectively, P≤0.05, LSD=0.175) and also E-L 9 (6.0 and 6.2 and 6.8 and 7.5 weeks, respectively, P<0.05, LSD=0.516), but for plants harvested in July, bud growth to E-L 4 and E-L 9 for both HWT and non-HWT was similar (4.0, and 4.1 for E-L 4 and 5.2 and 5.7 for E-L 9, respectively).

The month x cold storage interaction revealed a similar trend to month x HWT interaction in that time taken for cold stored plants harvested in June and May to reach E-L 4 was less than for non-cold stored plants, being 4.3 and 4.2 versus 5.1 and 6.7 weeks, respectively (P<0.05, LSD=0.295), and also to reach E-L 9 (5.8 and 5.4 versus 6.9 and 8.4 weeks, respectively, P<0.05, LSD=0.516).

The month x variety (P=0.008) interaction for bud growth indicated that 101-14 cv. Sauvignon blanc plants from the July harvest were fastest to reach E-L 4, with May, June and July times being 5.5, 4.6 and 3.9 weeks, respectively (P≤0.05, LSD=0.214) compared to 101-14 cv. Pinot noir (5.1, 4.4 and 4.0 weeks, respectively) and 5C cv. Sauvignon blanc (5.6, 5.0 and 4.2 weeks, respectively). The interaction was no longer significant by growth stage E-L 9 (P=0.085).

The interaction between HWT and cold-storage (P<0.001; Figure 4.12) indicated that cold stored plants were unaffected by HWT and were faster to reach E-L 4 than non-cold-stored plants (4.1 and 4.2 weeks, respectively, for HWT and 4.6 and 6.0 weeks, respectively, for non-HWT), whereas non-cold-stored plants which were slower overall, were also slower to E-L 4 with HWT than non-HWT. This pattern of effects was similar for times taken to reach E-L 9 (5.4 and 5.7 weeks and 6.2 and 7.6 weeks, respectively).

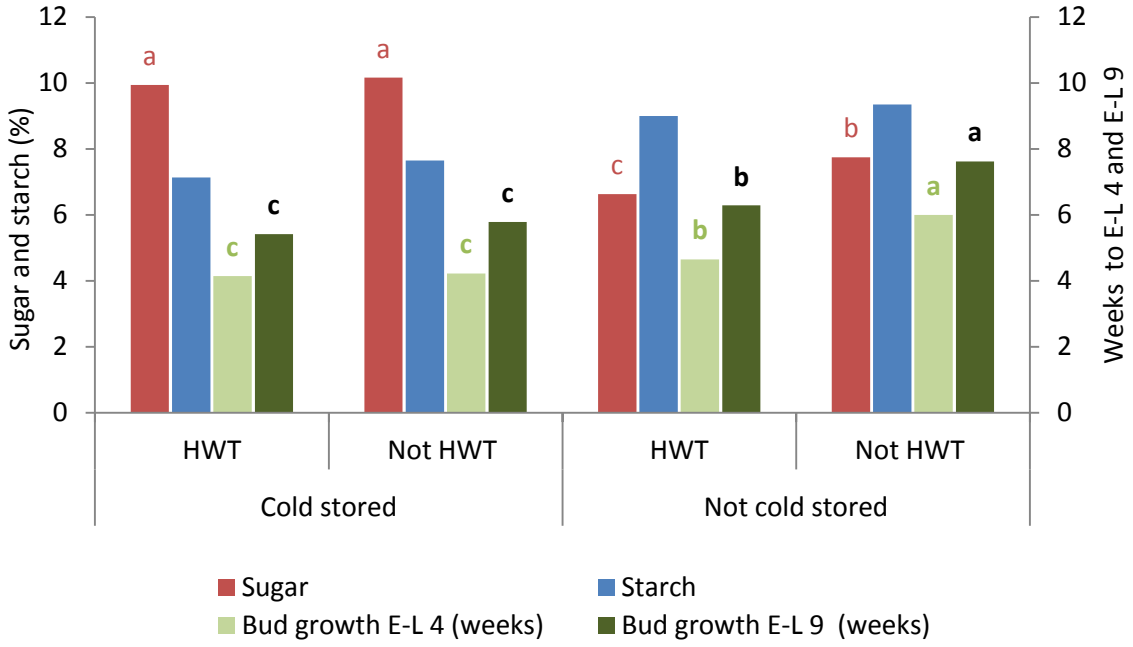


Figure 4.12 Mean percent sugar and starch in grapevine roots and weeks for buds to reach growth stage E-L 4 and E-L 9 for plants that were HWT and cold stored. All factors were significant (P≤0.05) except starch (P=0.686). Columns which are the same colours, red for sugar (LSD=0.953), blue for starch, light green for bud growth to E-L 4 (LSD=0.170) and dark green for E-L 9 (LSD=0.421). Columns with the same letters above are not significantly different (Fishers protected LSD P<0.05).

The HWT x variety interaction (P=0.004) indicated that after HWT, all varieties reached E-L 4 at similar times, but without HWT 101-14 cv. Pinot noir reached EL-4 more quickly (4.8 weeks) than 101-14 cv. Sauvignon blanc (5.1 weeks) and 5C cv. Sauvignon blanc (5.4 weeks,

LSD=0.175). While the above interactions were initially significant at growth stage E-L 4 they were not by growth stage E-L 9 (P=0.967).

The cold storage x variety interaction (P=0.001) indicated that after cold storage all varieties reached E-L 4 at similar times, but without cold storage 101-14 cv. Pinot noir reached E-L 4 more quickly (4.9 weeks) than 101-14 cv. Sauvignon blanc (5.3 weeks) and 5C cv. Sauvignon blanc (5.6 weeks, LSD=0.175). Again the interaction was no longer significant by growth stage E-L 9 (P=0.088).

There was a highly significant three way interaction between month of harvest, HWT and cold storage on growth to E-L 4 (P<0.001) and to E-L 9 (P=0.015) (Figure 4.13). The interaction was mainly associated with the greater time needed for May-harvested plants that were not cold-stored to reach E-L 4 and E-L 9, being highest for non-HWT (7.8 and 9.2 weeks, respectively) and HWT plants (5.5 and 7.6 weeks, respectively) compared to cold-stored plants (4.3 and 5.8 and 4.0 and 4.9 weeks, respectively).

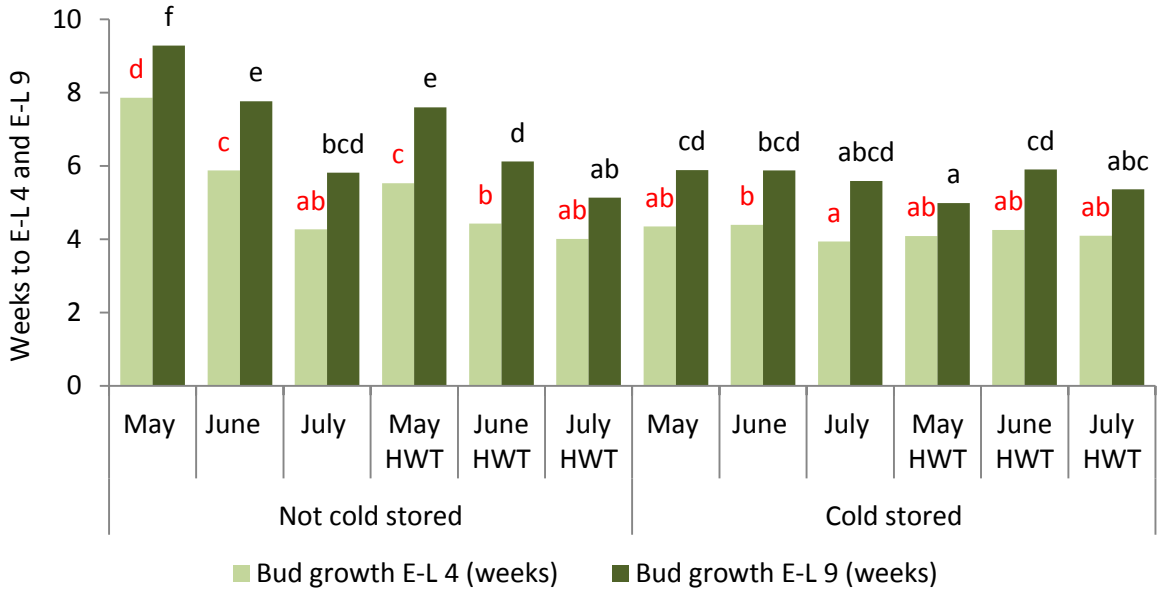


Figure 4.13 Weeks taken for bud development to stage E-L 4 and E-L 9 for plants harvested in different months showing the effects of HWT and cold storage (interactions significant to P<0.001 and P=0.015, respectively). Columns which have the same letters above (red for growth to E-L 4, LSD=0.417 and black for growth to E-L 9, LSD=0.729) are not significantly different (Fishers protected LSD P<0.05).

For June-harvested plants, bud growth to E-L 4 and E-L 9 was significantly slowest in non-cold stored plants which received no HWT, while the other treatments were similar. For July-harvested plants, bud growth to E-L 4 and E-L 9 was similar for all treatments (Figure 4.13).

4.3.2.2 Effects on root carbohydrates

Percent sugar in grapevine roots was affected by month of harvest (P=0.023) (Figure 4.10), mainly due to the difference between June and July data, both of which were not statistically different to May data (9.0, 8.2 and 8.6%, respectively, LSD=0.837). Percent starch was significantly greater in plants that were harvested in May than June and July (9.8, 7.7 and 7.3%, respectively, LSD=1.142).

There was a significant HWT effect on percent sugar (P≤0.001) but not starch; concentrations of both were greater in grapevine roots of non-HWT plants than HWT plants (8.9 and 8.2%, respectively LSD=0.674 and 8.5 and 8.0%, respectively, P≥0.05). Cold storage had a significant effect on percent sugar and starch (both P<0.001). Sugar concentration was greater in grapevine roots of cold stored plants than those which were not cold stored (10.0 and 7.2%, respectively, LSD=0.674). Conversely, percent starch was significantly greater in plants that were not cold stored than in cold-stored plants (9.2 and 7.4%, respectively, LSD=0.749).

There were no interactions between starch concentration and treatments (P≥0.05). However, there was a significant interaction between cold storage and HWT for percent sugar (P=0.025, Figure 4.12). Plants that were not cold stored and were HWT, had significantly less sugar than for the remaining treatments (Figure 4.12).

There was a significant interaction between month x HWT x cold storage for percent sugar (P=0.041) but not percent starch (P=0.306). For plants that were harvested in May, the concentration of root sugar was greatest in roots that were cold stored, irrespective of the HWT treatment (Figure 4.14). The lowest root sugar concentration was in plants that were not cold stored and received HWT. For June-harvested plants, percent sugar was also greatest in cold stored plants regardless of whether they were HWT, and was significantly more than for non-cold stored plants regardless of HWT (Figure 4.14). For July-harvested plants, cold stored plants without HWT had higher sugar concentrations than the non-cold-stored plants irrespective of whether they were HWT treated (Figure 4.14).

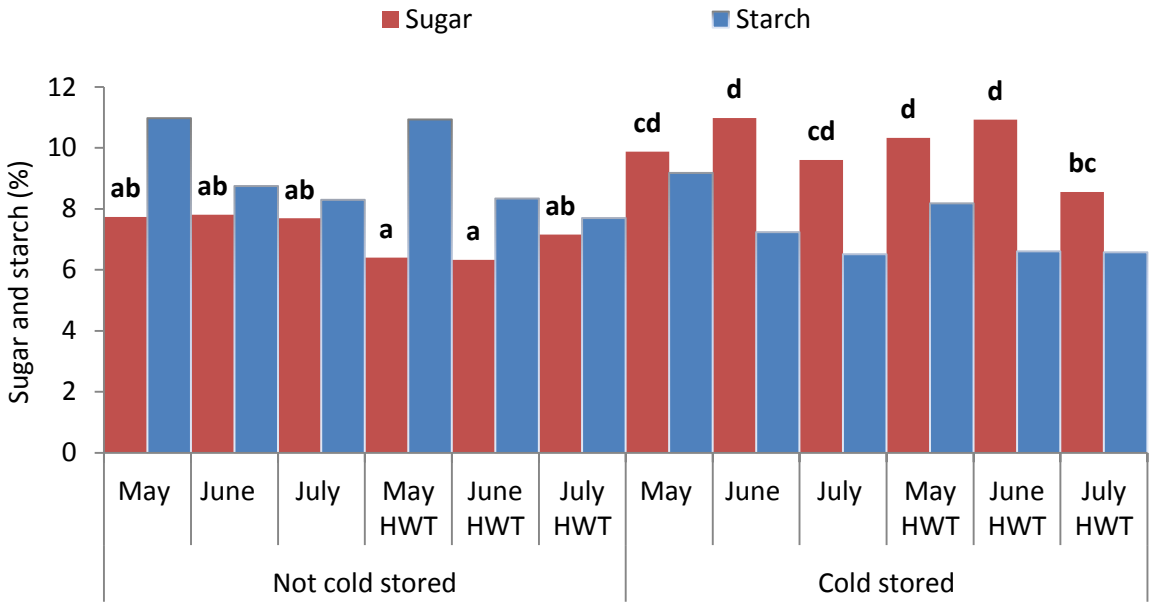


Figure 4.14 Percent sugar and starch in roots of plants harvested in different months, showing the effects of HWT and cold storage (interactions significant for sugar P=0.041 but not starch P=0.306). Columns which have the same letters above (red for % sugar LSD=1.652 are not significantly different) (Fishers protected LSD P<0.05).

The overall trends were for greater concentrations of root sugars in cool-stored plants harvested before July. For non-cold stored plants, there was a trend for reduced sugars in those treated with HWT although it was not significant (P≥0.05).

4.4 Discussion

This study showed that the month of harvest, HWT, cold storage and interactions between treatments had significant effects on the WSC content of root samples, bud development and *Cylindrocarpon* spp. infection for grafted grapevine varieties 101-14 cv. Pinot noir, 101-14 cv. Sauvignon blanc and 5C cv. Sauvignon blanc.

Disease incidence and severity reduced over time as plants harvested earliest had the most infection. Incidence and severity were also greatest in rootstock variety 101-14 cv. Pinot noir. The interaction between month and HWT was most likely due to the greater proportional effectiveness of the HWT (48.5°C for 30 min) on the 101-14 cv. Pinot noir plants harvested in May. HWT significantly reduced disease incidence compared to control plants and this concurs with the findings in Chapter 2 and 3 where HWT greatly reduced viability of

Cylindrocarpon pathogens directly and within rootstock cuttings. In this study, the grapevines were planted into soil which had been infested with *Cylindrocarpon* spp. and so subjected to relatively high disease pressure. However, the concentration of soil inoculum was clearly not abnormal since similar incidence of infection has been reported for dormant grafted vines lifted after 6-9 months in nursery soils. Young rooted vines had 50% infection incidence in a South African study (Halleen et al., 2003), 24% incidence in a Spanish study (Armengol et al., 2001) and 37% incidence in a Portuguese study (Rego et al., 2000).

The reducing disease incidence observed for plants harvested from May to July in this study was surprising since all plants had been treated in a similar manner in the field. The reason for that reduction was not likely to be due to reducing temperatures as winter advanced, because there was not a similar incidence reduction in cold storage, where the temperature (2-3°C) was likely to be colder than the soil temperature in July. Little is known about the effect of plant dormancy on fungal community dynamics but fungi have developed genetic capability and biochemical mechanisms for life-style conversions, such as to sclerotia (Rodriguez and Redman, 1997) and for *Cylindrocarpon* spp. to chlamydospores (Booth, 1966). These structures are able to accommodate dynamic environments although the processes are difficult to gauge in their natural environments (Rodriguez and Redman, 1997). In this study disease incidence at the base of the grapevine trunk (0 cm), significantly reduced from May to July which coincided with the onset of dormancy, however, disease incidence at 5 cm further up the trunk was similar for May, June and July and so may indicate a slowing of the endophytic activity. The reducing incidence was reflected by a reduction in root starch concentration during the harvest months, but there is little evidence to support a relationship between root disease and changes in CHO concentrations. Stress related CHO changes have been reported, although without reference to disease incidence (Colhoun, 1973; Wargo and Harrington, 1991).

Plant predisposition to disease development is influenced by environmental stresses such as unsuitable water and temperature conditions, defoliation, transplant shock, lack of nutrients and disease pressure (Scheck et al., 1998; Schoeneweiss, 1975). Scientists have reported relationships between stress, CHO reserves and plant hardiness (Carroll et al., 1983; Colhoun, 1973; Schoeneweiss, 1975, 1981; Wargo and Harrington, 1991) and shown that increased plant hardiness in woody plants that survived freezing temperatures often

resulted in defence mechanisms which reduce disease susceptibility (Schoeneweiss, 1975). In sugar maple trees, defoliation stress induced conversion of starch to reducing sugars and made the sugar maple tree more vulnerable to attack by *Armillaria* pathogens, which were better able to utilise reducing sugars than sucrose (Wargo, 1991). However, the reduction in starch was not matched by an increase in sugar in this study and the concentrations of reducing sugars were not analysed. The reducing disease incidence during winter more probably indicates death of the pathogen in some of the later-harvested plants, although this is hard to explain.

The negative effects of grapevine diseases are generally well appreciated (Scheck *et al.*, 1998) but the metabolic interactions between pathogens and hosts are not well understood These interactions might include defence mechanisms or the involvement of toxins produced by trunk disease fungi however, this was not investigated in this research. Andolfi *et al.* (2011) reported that phytotoxins were produced by pathogens associated with grapevine trunk diseases and these could be further investigated in respect of *Cylindrocarpon* spp. They showed that leaves infected with esca pathogens had increased phenolic compounds and less reducing sugars compared to non-diseased leaves. The toxins produced by esca and *Botryosphaeriaceae* pathogens caused symptoms in the wood where they were produced and also in leaves. Whether the toxins were translocated to leaves or produced on site in response to signalling mechanisms was not known but the number and composition of toxins in the leaves were reported to be influenced by the physiological state of the vine and environmental conditions, such as rainfall and temperature. They concluded that further research was needed to link the mode of action, environmental influences, disease development and symptom expression (Andolfi *et al.*, 2011) which may provide an explanation for the reduced starch and infection over time, shown in this research.

In the current study, infection appeared to be influenced by the cultivar Pinot noir, because when Pinot noir was grafted to rootstock 101-14 *Cylindrocarpon* infection levels were significantly greater than in 101-14 grafted to Sauvignon blanc. Although, the studies in Chapters 2 and 3 with rootstock cuttings showed that 101-14 was more susceptible to black foot disease than 5C, disease incidence was similar in rootstocks 101-14 and 5C when grafted to Sauvignon blanc in this study. The colonisation by *Cylindrocarpon* spp. appeared to progress more readily through the xylem tissue of rootstock 101-14 cv. Pinot noir than the

other two combinations since disease incidence measured at 5 cm was significantly greater in that variety. The greater disease incidence in rootstocks grafted to Pinot noir may be associated with this scion cultivar; it is a difficult cultivar to grow with delayed and stunted growth (Barr, 1992; Waite *et al.*, 2006a) which may stress the rootstock. This could have influenced disease development in rootstock 101-14 as the way in which rootstock and scion interact is not well understood (Gu, 2001). Komar *et al*. (2010) investigated the effects of leaf roll and the 'rugose wood disease complex' viruses on *V. vinifera* 511 clones of Savagnin rose that were grafted to three rootstocks: *V. rupestris,* Kober 5BB and 161-49 Couderc. The viruses did not appear to cause significant yield or production effects and the rootstocks appeared to 'compensate' for loss of production caused by the viruses in affected Savagnin rose cultivars (Komar et al., 2010). The current study demonstrated the greater susceptibility of the combination of rootstock 101-14 with cv. Pinot noir to *Cylindrocarpon* infection so it was unfortunate that the experiment did not include a fourth combination of 5C cv. Pinot noir as that would have provided greater insight and evidence into rootstock and cultivar interactions and disease incidence. Despite the increased infection in 101-14 cv. Pinot noir, there was no difference in *Cylindrocarpon* infection between the three varieties after HWT and disease incidence was significantly reduced compared to non-treated plants (5.5 and 32.4%, respectively). Further investigation into rootstock and cultivar interactions could be beneficial to establish combinations that may have greater resistance to trunk disease pathogens especially when planting into at risk soils.

Grapevine roots accumulate CHO during carbon and nitrogen transfer from the leaves before abscission, which in this trial occurred during May and June (Hunter et al., 1995; Scholefield et al., 1978). Carbohydrates are usually highest at the end of the growing season and decrease during dormancy (Winkler and Williams, 1945). Winkler and Williams (1945) reported that the reduction of root starch concentration in grapevines during winter was likely to be caused by starch utilisation for 'slight growth' or increased respiration and not the conversion of starch to sugar, which in their study remained 'fairly constant'. In the current research, starch concentration did reduce between May and July harvests which indicated some utilisation of starch and so concurred with Winkler and Williams (1945). However, the end of the growing season differs between countries and in cool climates like New Zealand, it usually coincides with harvest and often frost (Bennett, 2002), whereas in the absence of frost in warm climates like California, leaf fall is usually several weeks after

harvest (Williams, 1996). In these countries, the patterns of CHO accumulation and depletion may therefore be different to that shown in the current study.

Dormancy is induced in grapevines by prolonged exposure to short days and/or low temperatures (Andreini et al., 2009). In the current study, the average maximum temperature decreased during May, June and July (17.2, 16.3 and 15.0°C, respectively, Appendix E.10.1), with the first frost recorded in June and then on four consecutive days in July (NIWA, 2012). Freezing temperatures have also been correlated with reduced root starch concentrations and increased soluble sugar used to protect plant tissue from freezing or cold temperature damage (Hamman et al., 1996; Williams, 1996) however there did not appear to be a link in the current study since the difference in root starch concentration from May to July across all treatments was much greater than the differences in sugar concentration which remained relatively constant between harvest dates and within a treatment, and so concurred with Winkler and Williams (1945). The starch concentration constantly reduced from May to July which was consistent across all treatments hence there was no significant starch interaction.

From a study over consecutive years from autumn to spring, Carroll *et al*. (1983) reported that unhealthy sugar maple trees stressed by drought, site disturbance and road salt had 80% lower or depleted starch than 'normal' trees and the greatest dieback. Although their study did not investigate a relationship between reduced starch and disease incidence their findings indicated that plants under stress had reduced starch concentrations, which was in contrast to the current study where grapevines with the greatest starch concentration (May) had the most disease but as starch reduced to July so did infection. Horsley *et al*. (2000) reported that in stressed sugar maple trees, starch was converted to reducing sugar and made the trees vulnerable to pathogen attack by secondary organisms like *Armillaria mella* (Vahl.) Quel. possibly because those pathogens were able to utilise reducing sugars such as fructose and glucose better than sucrose (Wargo, 1991). Further investigation could be conducted to investigate a potential relationship between disease development and CHO concentrations.

The differences in starch and sugar concentrations in root samples of HWT and non HWT plants, although significant, were small. However, in cold stored plants there were much higher concentrations of sugar and lower concentrations of starch than for non-cold stored

plants, which seemed to indicate that starch was converted to sugar in cold stored plants but not in non-cold stored plants. Waite *et al*. (2006b) reported that during cold storage grapevine plants generally displayed increased respiration for the first few weeks followed by quiescence. However, they reported that Pinot noir cuttings continued to respire aerobically after HWT which may explain the reduced CHO content in roots samples of 101-14 cv. Pinot noir in this study. The increased respiration during early stages of cold storage may also provide some explanation for the significant overall reduction observed in root starch content of cold stored grapevines compared to non-cold stored plants and this gradual starch depletion concurs with Winkler and Williams (1945).

In Australia, Waite *et al*. (2006b) investigated some effects of HWT (50°C for 30 min), hydration (8 h) and cold storage (4-5°C) on the metabolism of dormant one year old, two bud cuttings of *V. vinifera* varieties Pinot Noir and Cabernet Sauvignon, which were the most and least sensitive, respectively, to HWT (Waite *et al.*, 2006b). All plants were cold stored in perforated plastic bags and after four weeks cold storage ethanol production was measured from cuttings held in sealed test tubes. Aspirated samples were taken by syringe and the gases analysed by gas chromatography (Waite *et al.*, 2006b). They found that 24 h cold storage after HWT caused increased respiration rates that were significantly higher for HWT cuttings, plus or minus hydration, than cuttings that were not HWT or hydrated only. However, after four weeks cold storage, anaerobic respiration was reduced and similar for all plants. Even though cold storage can induce low respiration (Waite *et al.*, 2006b), the authors reported that the use of well ventilated bags was necessary to allow drying of plant tissue to avoid fermentation thereby improving plant quality and reducing plant mortality. That study also supported the results of the current research in which cold storage had a positive effect on bud development, regardless of whether plants were HWT or not.

Hydration of grapevine material was reported to cause plant failure and increased disease levels as a result of microbial contamination from hydration tanks (Cole and Waite, 2006; Waite and May, 2005; Whiteman, 2004), and although the reason for plant failure was unclear, the discontinuance of hydration was recommended (Waite and Morton, 2007). However, hydration is still used by many nurseries to compensate for dehydration of plants when their processing was delayed in nurseries. In the current study grapevines were subjected to a 10-15 min dip in cold water prior to HWT which is a much shorter hydration

than the standard practice of soaking propagating material overnight (Waite and May, 2005). It is possible that hydration may compromise the physiological process of dormancy since 'completely dormant' grapevines have extremely low water content which suppresses metabolism and can be activated by hydration (Lavee and May, 1997). If so, hydration may disrupt dormancy of grapevines or cuttings that are not completely dormant such as those harvested very early in winter or during late winter/early spring, however, the very short hydration period used in this study is unlikely to have affected dormancy. The research by Waite and Morton (2007) led to modification of the protocols of Corbans Viticulture Ltd for propagation of plants which were then cold stored before HWT and the change has resulted in more high grade plants and less mortality (A. Graham pers comm. 2008).

The current study, in which HWT (50°C for 30 min) plants were soaked in cold water for 30 min after treatment had zero plant mortality, differed from research by Gramaje and Armengol (2012) in Spain. They investigated HWT (53°C for 30 min), hydration and cold storage with grafted plants of *V. vinifera* cultivar Tempranillo grafted to rootstock 110 Richter plants. All plants that were HWT were soaked in water for one hour prior to HWT, however some plants were not given a cold soak treatment after HWT, which is current practice to avoid heat damage to plant tissue after HWT. The post HWT practice of rapidly cooling grapevine material in cold water is under review (Waite *et al.*, 2012), however, the removal of that step in the study by Gramaje and Armengol, (2012) resulted in significantly greater plant mortality, seen as non-emergence of buds which Wample (1993) associated with mortality. Plant viability was 98.5, 88.7 and 70% for plants cold-stored for 1, 2 and 4 weeks after HWT, which indicated they had suffered heat damage from HWT that was exacerbated by the longer periods of cold storage. After 10 months growth, the HWT and non-cooled young vines that were viable had significantly reduced shoot length (32.0, 20.5 and 11.9 cm, respectively for 1, 2 and 4 weeks cold-storage) and weight (8.5, 9.0 and 5 g, respectively) than control plants (42.5 cm and 13 g, respectively), questioning the rationality of eliminating the post HWT 30 min cold water soak. That study also indicated that prolonged cold storage of vines after HWT at 53°C for 30 min was detrimental to the health of young vines, unlike this research in which bud growth was not significantly slower for cold stored vines, but these vines were cold stored and then HWT.

HWT cuttings have been reported to establish more slowly than non HWT cuttings (Waite, 2005; Waite *et al.*, 2006a) but this research which is the first to measure interactions between HWT, cold storage, bud development and CHO concentrations of field grown grafted grapevines showed HWT grapevines reached growth stages E-L 4 and E-L 9 earlier than non-HWT plants. Bud growth to E-L 4 and E-L 9 was also quicker for plants that were cold stored than non-cold stored plants. However, regardless of treatment, over all months bud growth was similar for plants harvested in July.

Over all months, the untreated control plants took almost two weeks longer to reach growth stage E-L 9 and were slower than plants that were HWT but not cold stored. These effects were greater for plants harvested in May and June than July. This result differed to the results of Waite (2002) who reported that bud and root development of HWT cuttings was initially delayed but recovered during the season. In the current research, bud break data to growth stage E-L 4 was analysed and showed similar trends to the results measured at growth stage E-L 9 except that interactions between varieties and treatments were initially significant but not significant by growth stage E-L 9.

Andreini *et al*. (2009) reported that bud break was likely to be influenced by roots and their phytohormones and effects varied according to the cultivar (Prakash and Reddy, 1990). In addition, Sabbatini *et al*. (2012) reported that bud growth was principally influenced by the scion. The current research appeared to agree with both statements since cultivar Sauvignon blanc grafted to rootstock 101-14 broke bud and reached growth stage E-L 9 earlier than when it was grafted to rootstock 5C, which supported a rootstock effect. In addition, Pinot noir grafted to 101-14 broke bud and reached growth stage E-L 9 earlier than Sauvignon blanc grafted to 101-14, which indicated a scion effect. It also illustrates the complexity of interactions between rootstock and scion, which may also be influenced by different environmental conditions due to annual and regional variations (Sabbatini *et al.*, 2012).

Waite *et al.* (2006a) reported that Pinot noir was more sensitive to and adversely affected by HWT than Cabernet Sauvignon cuttings. They suggested that the subsequent variability in growth between Pinot noir and Cabernet Sauvignon cuttings after HWT and hydration was due to damage of starch grains (amyloplasts) caused by HWT or hydration, but damage was similar in both varieties and therefore did not explain the delayed growth of Pinot noir. However, in the cuttings that had been cold stored for four weeks the damaged amyloplasts

'appeared normal and undamaged' in both varieties which indicated recovery during cold storage (Waite *et al.*, 2006c). In the current study, bud break was affected by month, HWT, cold storage and there were interactions between those factors but there was no interaction with variety which implied that differences in bud break between varieties were influenced by rootstock and scion phenology rather than the treatments. Results in this study indicated a link between higher sugar levels after cold storage and quicker bud break. However the month x HWT x cold storage interaction showed that for plants harvested in July, sugar concentration in root samples of cold stored and HWT plants was similar to those in non-cold stored plants, and that bud growth to E-L 9 regardless of treatment was similar and much quicker than for May and June non-cold stored plants.

A study for which the results agreed with those of the current research was conducted in Washington by Wample (1993). He investigated use of HWT (52, 54, 56, 58 and 60°C for 10, 20 and 30 min) and cold storage (3-4°C), pre HWT and post HWT for four weeks, for control of crown gall disease of grapevines. After HWT and cold storage Cabernet Sauvignon cuttings were planted into moist peat moss and grown in a green house for nine weeks during which time bud break was evaluated twice weekly. Over all treatments, he reported that days to bud break (DBB) and plant mortality increased with increasing HWT temperatures and times, and DBB were least in cuttings that had been HWT then cold stored. Bud break was earliest in plants harvested in January and slowest in those harvested in November, which also concurred with this study equating to July and May, respectively. Plant mortality was associated with the time of harvest and treatment, and was greatest in HWT cuttings that were harvested in February which the author attributed to their loss of dormancy as spring approached and therefore, lower thermotolerance. Interestingly, plants harvested in November and HWT at the suggested optimum temperature of 56°C for 30 min (Wample, 1993) and then cold stored had much greater mortality than those that were cold stored then HWT (100 and 20%, respectively). Since plants harvested in January and then given the same treatments, had mortality rates of 10 and 20%, respectively, this indicated that plants harvested very early in the season should be cold stored prior to HWT to reduce mortality. Wample (1993) was not able to determine whether the difference in days to bud break between treatments was due to HWT or cold storage but recommended that to control crown gall disease, cuttings be HWT after late harvest and then cold stored.

This research showed that for plants harvested in May and June, whether HWT or not, the time taken to reach growth stage E-L 9 was generally greater for non-cold stored than for cold stored plants. However, there was also a HWT effect for non-cold stored plants which was dependent on the month of harvest, so non-cold stored plants harvested in May were significantly slower to reach growth stage E-L 9 than May cold stored and HWT plants, which was also the case with plants harvested in June but not July, and this did not appear to be influenced by sugar concentrations which were similar for all treatments.

Lavee and May (1997) also found that cold chilling for 10 days at temperatures below 10°C was needed to release dormancy in the grape cultivar Merlot and that budburst of non-chilled grapevines was less frequent and more variable (Lavee and May, 1997). However, Waite *et al.* (2006c) reported that cold storage did not induce deeper dormancy in grapevine cuttings and Wample (1993) reported that bud burst was not stimulated in HWT cuttings after removal from cold storage. Since HWT did not increase mortality or time taken to bud break in this study, it agrees with the conclusion of Waite *et al*. (2006b) that excessive hydration, use of non-perforated storage bags during cold storage and use of substandard or damaged cuttings are more likely to cause poor quality propagation material than HWT. In fact, this study showed beneficial effects of HWT on plant growth since bud break was earlier in HWT plants than non-HWT grapevines. Disease in the field grown grafted grapevines did not increase during cold storage and grapevines that were harvested latest had the least infection therefore it would seem prudent to harvest plants when they were most dormant and if not practical, plants that are not completely dormant should be cold stored for a period of four weeks prior to HWT.

In reviewing the literature around HWT it is pertinent to note that much of the research has been conducted on *V. vinifera* cuttings, and often one variety, unlike this research which used one year grafted grapevines. Also results have varied between countries which are most likely due to differing HWT methods or may be due climatic and environmental differences.

Chapter 5

Biofumigation and rotation crops for control of *Cylindrocarpon* species

5.1 Introduction

Crop rotation is a well-established cultural practice used to maintain healthy soil and control soil-borne diseases particularly in annual monoculture crops such as onion, pea, potato, tomato and wheat (Chaube and Singh, 1991). Monoculture in a cropping system provides a stable environment and host for pathogens, whereas more diverse cropping systems provide for greater competition by supporting a wider range of soil flora (Raaijmakers *et al.*, 2009). Therefore introducing greater host diversity with different crops by rotational cropping can increase the diversity of soil flora and affect the suitability of the soil environment for pathogen survival and development. Crop rotation can reduce disease levels as it causes disturbance of the host, environment or pathogen, all the components that need to be optimum for development of a disease. Chaube and Singh (1991) reported that disease control by crop rotation occurs through starvation of the pathogen by changing the host plant, increasing variation in microbial activity and antagonism, and changing the physiochemical environment of the soil. Some alternative host plants can release toxic plant substances which are inhibitory to the pathogen, a process referred to as 'biofumigation' when it involves the use of brassicaceous crops, which are particularly effective against some soil-borne pests and pathogens (Kirkegaard *et al.*, 1993).

Brassicaceous crops incorporated into the soil release isothiocyanates (ITCs) which are known to suppress pathogenic fungal species. *Brassica* species contain significant quantities of the thiosglucoside compounds known as glucosinolates (GSLs). When GSLs are hydrolysed by the myrosinase enzyme present in *Brassica* tissue, volatile isothiocyanates are produced (Kirkegaard and Sarwar, 1998). Isothiocyanates are known to have broad biocidal activity in the suppression of pathogenic fungal species, nematodes, weeds, and some insect species (Brown and Morra, 1997). The principal suppressive effect of brassica amendments to soil-borne diseases occurs at flowering, immediately after their maceration and incorporation

into soil but also during decomposition (Mattner *et al.*, 2008) and during growth when ITCs are exuded from the roots of the plants.

In *in vitro* and/or field trials, *Brassica* species have been reported to reduce the activity of soilborne pathogens of potato (Larkin and Griffin, 2007), sugarcane (Pankhurst *et al.*, 2005), carrots (Montfort *et al.*, 2011) and Douglas-fir nursery seedlings (Smolinska *et al.*, 2003). However, Stephens *et al*. (1999) found that biofumigation with Indian mustard did not significantly reduce incidence of soil-borne *Cylindrocarpon* spp. in roots of nursery grapevines and significantly increased incidence of *Pythium* spp., thereby increasing mortality in the grapevine nursery stock. The aim of this study was to determine the efficacy of oats as a green manure crop and *Brassica* spp. for biofumigation control of *Cylindrocarpon* spp. in grapevines with *in vitro* and *in vivo* experiments.

5.2 Preliminary experiment with different rotation species

5.3 Materials and methods

A preliminary experiment was conducted at a Lincoln site (HRA) to test the efficacy of three rotation crops with different properties on infection by *Cylindrocarpon* spp. from soil inoculum. The crops included oats (*Avena sativa*, seed cultivar unknown, supplied by AgriQuality, Lincoln), which is commonly used as an inter-row species in New Zealand vineyards, and two types of brassicas with different growth habits: mustard (*Brassica juncea*, seed supplied by Smiths Seeds Ltd, Ashburton) and rape (*B. napus*, seed cultivar unknown, supplied by AgriQuality, Lincoln). The experimental site was in an area that had previously been inoculated with mixed conidial inoculum made from nine isolates, three each from *C. liriodendri*, *C. macrodidymum* and *C. destructans* (Section 2.3).

5.3.1 Rootstock material

The grape rootstock varieties 101-14 and 5C supplied as dormant cuttings by Corbans Viticulture were prepared as described in Section 2.8.3.

5.3.2 Treatments

The experiment was planted in October 2007 and was arranged in a completely randomised split plot (rootstock varieties 101-14 and 5C) design with five replicate blocks, each

containing eight plots of 500 mm separated by 200 mm buffer zones. The four treatments were: (Trt 1) mustard, (Trt 2) rape, (Trt 3) oats and bare land control (Trt 4). The soil was rotary hoed and the surface raked to give a fine tilth before seeds were broadcast (15 g/m) then lightly raked and irrigated for 30 min by a Vyrsa 20 mm impact sprinkler (Alister Bevan Products Ltd, Christchurch), the site was further irrigated for 30 min on alternate mornings. The oats (Figure 5.1a) and brassicas were grown until the mustard and rape plants had reached 50% anthesis, at approximately five weeks, and then all rows were rotary hoed (Figure 5.1b), which chopped and incorporated plant material into the soil. The T-Tape irrigation system was laid on the soil of each row and the rows were covered with polythene as described in Section 2.10 (Figure 5.1c) before irrigation began.

Figure 5.1 Plots of oats grown for 5 weeks *in situ* (a), rotary hoeing of crops of mustard, rape and oats into the soil (b) polythene covered rows planted with rootstocks 101-14 and 5C two weeks after incorporation of crops (c).

5.3.3 Field site preparation

After two weeks, the callused rootstock cuttings were planted through the polythene into the treated and bare land plots (Figure 5.1c) according to standard nursery practice. In brief the rootstock cuttings were planted in double rows of six plants with 100 mm between each plant, giving 12 plants per plot (Figure 5.1c) and 200 mm between plots. Only the centre eight plants of each plot were assessed for *Cylindrocarpon* spp. infection as described previously (Chapter 2). The site was irrigated for 30 min each day by T-tape, being controlled by an electronic timer as described in Chapter 2.

5.3.4 Assessment

The plants were grown for 9 months, then harvested and infection assessed as described previously (Section 2.9.1).

5.3.5 Statistical Analysis

The data of *Cylindrocarpon* spp. disease incidence per plot (from 0 cm and 5 cm isolations) and severity (the mean proportion of tissue pieces infected at 0 cm per plot) were analysed by general linear model (GenStat Release 14.1, VSN International Ltd, U.K.) with terms appropriate to the design and the two-way interactions amongst the factors of interest. If significant main effects or two-way interactions were identified, the significance of differences between means from individual treatments was further explored using standard errors or Fisher's protected LSD tests. A P-value of ≤0.05 was taken to indicate statistical significance.

5.4 Results and Discussion

The treatments did not have a significant effect on percent disease severity and incidence in plants at 0 cm and 5 cm (P=0.267, P=0.338 and P=0.537, Appendix F.1.2, F.1.1 and F.1.3, respectively). There were no significant variety effects for any treatments (P=0.599, P=0.196 and P=0.079, respectively, Appendix F.1.2, F.1.1 and F.1.3).

There was, however, a trend for reduced infection in plants of rootstock 101-14 and 5C that had been planted into soil amended with mustard plants (Figure 5.2) compared to control plants, since disease incidence (0 and 5 cm) and severity were reduced by 25, 65 and 33%, respectively. Unexpectedly, infection was generally greater in plants that were planted into soil amended with oats and rape than control plants (Figure 5.2).

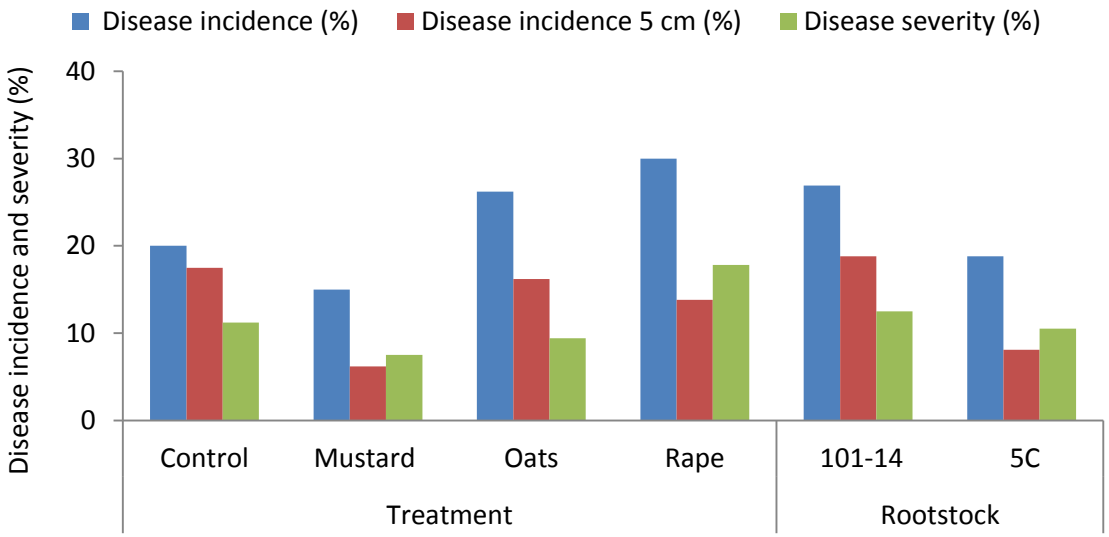


Figure 5.2 Mean percent disease incidence (combined for 0 cm and 5 cm) and at 5 cm, and disease severity in all plants from soil amended with mustard, oats and rape and an untreated control (P>0.05) and mean disease incidence (combined for 0 cm and 5 cm) and at 5 cm, and disease severity for rootstock 101-14 and 5C (P>0.05).

Oats were reported as ineffective against soilborne potato diseases in two field experiments (Larkin and Griffin, 2007) when they were planted as a rotation crop. In their repeated field experiments, oats were not significantly effective for reducing the incidence and severity of powdery scab or black scurf of potatoes. However, green manure crops of Indian mustard (*B. juncea*), rapeseed (*B. napus*), canola and ryegrass at one site and canola, rapeseed and yellow mustard (*Sinapis alba*) at the second site reduced incidence and severity by 16-40 and 48-78%, respectively, with mustard being the most effective treatment against powdery scab and common scab diseases, and rapeseed and canola being most effective in reducing black scurf disease (Larkin and Griffin, 2007). Rapeseed and canola seed have similar components, however, they differ in their chemical or fatty acid profiles. Since rapeseed contains at least 45% erucic acid it is used mostly for industrial purposes compared to edible canola seed which has less than 2% (Boland, 2012). Breeding of rape varieties over the last decade has produced low glucosinolate levels in the plant material in an attempt to make it more digestible to humans and animals (Bones and Rossiter, 1996). In this study the rapeseed cultivar provided was not known and it was not effective against the *Cylindrocarpon* pathogens therefore it may have been a commercial cultivar very low in glucosinolates such as cultivar 'double low'.

Isothiocyanates released by different members of the *Brassicaceae* family vary between species (Clark, 2010) with over 120 ITCs identified (Fahey *et al.*, 2001). The GLS sinigrin (2-Propenyl C_3) was reported to occur in high concentrations in seeds and seedlings of mustard (*B. juncea*) but was absent in rape (*B. napus*) (Rangkadilok *et al.*, 2002), which had more abundant Glucobrassicanapin (4-Pentenyl C_4) (Clark, 2010). The ITCs released from *Brassica* species such as mustard, rape and canola were shown to be inhibitory to potato pathogens such as *R. solani* (Larkin and Griffin, 2007) but in the current study *Cylindrocarpon* pathogens appeared to be somewhat inhibited by mustard and not rape. That may have meant that *Cylindrocarpon* isolates were more susceptible to sinigrin, the predominate ITC found in mustard, however further investigation to validate that hypothesis would be required and was outside the scope of this research.

There was a trend for greater disease incidence in plants at 0 cm and 5 cm and severity in rootstock variety 101-14 than 5C (mean percent 26.9, 18.8 and 12.5, and 18.8, 8.1 and 10.5, respectively, Figure 5.2) which concurred with previous experiments (Chapters 2 and 3). From a biological perspective, the reduced disease levels in plants that that had been treated with mustard, compared to treatments with rape, oats and the control plants, justified further investigation.

5.5 Section 1 Biofumigation with mustard - *in vitro* assays

5.6 Materials and methods

Mustard (*B. juncea*) was tested in an *in vitro* experiment using chopped mustard plants which included the roots and shoots and finely ground mustard seeds (mustard meal) in dual cultures with *Cylindrocarpon* spp.

5.6.1 *Cylindrocarpon* isolates

The nine previously used isolates (Section 2.4.2) of *C. destructans* (1D, 2D and 3D), *C. liriodendri* (1L, 2L and 3L) and *C. macrodidymum* (1M, 2M and 3M) which had been maintained on SNA slants as described in Section 2.3.1, were tested in this experiment. The isolates were subcultured onto PDA plates and incubated at 20°C for 2-4 weeks when colonies had grown sufficiently to provide inoculum for the biofumigation experiments.

5.6.2 Mycelium growth from mycelial inoculum

For each of the nine *Cylindrocarpon* isolates, mycelium plugs (6 mm diameter) were cut from the growing edges of the 2-4 week old colonies grown on PDA and placed onto MEA in the centre of inverted deep-base (25 mm) Petri dishes, five replicate plates for each isolate and treatment. The six treatments were: chopped mustard plants (Trt 1), chopped mustard plants + soil (Trt 2), mustard meal (Trt 3), mustard meal + soil (Trt 4), soil control (Trt 5) and MEA control (Trt 6).

Figure 5.3 Mustard plants grown for five weeks in 4 L plastic containers (a), mustard plants chopped in a Multiblender™ (b), whole mustard seed that were ground to a fine meal in a mortar and pestle (c), soil and chopped mustard roots and shoots (d), finely ground mustard meal in Petri lid (e), incorporated soil and chopped whole mustard plants in Petri dish lid and *Cylindrocarpon* isolates placed into a 25 mm deep Petri dish (f) ground mustard meal in a Petri lid and *Cylindrocarpon* isolates in a 25 mm deep Petri dish (g).

The soil was collected several days prior to the experiment from the Lincoln field site (HRA), passed through a sieve of 4 mm pore size and stored in an air tight container to avoid desiccation. Later oven drying and weighing showed that it had a moisture content of ~80% of field capacity.

To grow the mustard plants required for the experiment, *B. juncea* seeds (5 g) were broadcast onto potting media (Section 4.2.2) in five 4 L plastic pots and grown in a greenhouse until 50% anthesis (Figure 5.3a). On the day of the experiment, the mustard plants were removed from the pots, their roots washed free of potting media and they were placed between damp paper towels until required. Sixty mustard plants (~120 g fresh weight) were placed into a MultiBlender™ (Sunbeam Corporation Ltd, China) with enough sterile water (~30 mL) to avoid clumping and pulsated for 20-40 seconds (Figure 5.2b).

For treatments one and two, approximately 2.5 g of chopped mustard plants were immediately placed on an inverted Petri lid (Trt 1) or incorporated into 50 g of soil on the Petri dish lid (Trt 2) (Figure 5.2d and f) and covered by the inverted base with agar and mycelium plug. For treatments three and four mustard seeds (0.5 g) were finely ground in a mortar and pestle (Figure 5.2c) from which 0.5 g was placed on an inverted Petri lid (Trt 3) (Figure 5.2e and g) or incorporated into 50 g of soil on the Petri dish lid (Trt 4), then immediately covered with the inverted Petri base and mycelium plug. For treatments five and six (controls) plates of MEA (Trt 5) and soil (50 g) (Trt 6) had no mustard material but were still covered with the inverted Petri base and mycelium plugs. The plates were sealed with cling film and randomly allocated to positions in a 20°C incubator for 7 days under a diurnal light schedule (12 h light, 12 h dark). Mean mycelium growth was assessed on each agar plate as the mean of the perpendicular colony diameters measured with a digital calliper (Mitutoyo, UK Ltd), less the diameter of each inoculating mycelium plug.

5.6.3 Germination and mycelial growth from conidial inoculum

The *in vitro* experiment on growth that incorporated conidium germination used the same nine *Cylindrocarpon* isolates and six treatments used to assess mycelium growth (Section 5.5.2) except that for Trt 3 the mustard meal was lightly sprayed with sterile water. This was because the plates for this experiment had been prepared with a very thin layer of agar to ensure germination could be observed, so the plates were much dryer than those used in the earlier mycelium experiment. Conidium suspensions were prepared for each of the nine

isolates by pipetting 5 mL of SDW onto 2-4 week PDA culture plates as described in Section 2.4.3 and then scraping the surface of each plate with a sterile hockey stick to dislodge the conidia. Each resulting suspension was strained through a 150 µm sterile sieve and poured into separate sterile Universal bottles containing 5 mL of SDW. The conidium concentration was adjusted to $2x10^2$ conidia/mL using a haemocytometer. Then 100 µL of each isolate's conidial suspension was spread over a thin layer of MEA in the base of a 25 mm deep Petri dish. The inverted Petri base was placed over its lid which contained the mustard treatment or controls as described (Section 5.5.2). The five replicate plates per treatment were sealed with cling film and randomly allocated to positions in a 25°C incubator overnight in dark, then removed and the colony forming units (CFU) that developed on each plate were counted. The plates were then randomly placed on a table for 5 days at ambient room temperature (20°C) before five randomly selected colonies for each isolate and treatment were measured with a digital calliper.

5.6.4 Statistical Analysis

The experimental design was a randomised factorial design with nine isolates, (three isolates from each of the three *Cylindrocarpon* spp.) and five replications for each of the experimental treatments. The data were analysed by general analysis of variance using SPSS version 17.0 (SPSS Inc., Chicago, USA) to determine biofumigation, species and isolate effects, and when significant effects occurred means were separated using Fisher's protected LSD at P≤0.05. An additional Dunnett t-test (2-sided) pairwise comparison was used to investigate the overall effects between all treatments and the control.

5.7 Results

5.7.1 Mycelium growth from mycelium plugs

There were significant effects of species (P≤0.001, Appendix F.2.2), isolates (P≤0.001, Appendix F.2.1) and treatments (P≤0.001, LSD=1.50, Appendix F.2.1) on colony diameters. Mycelium growth was significantly different for the three species, being greater for *C. destructans* than for *C. liriodendri* and *C. macrodidymum* (means of 25.6, 18.7 and 15.4 mm, respectively, LSD=1.40). There was also variation in mycelium growth across the isolates (LSD=1.83) of *C. destructans,* mean diameters for isolates 1D, 2D and 3D being 27.3, 19.8 and 29.7 mm, respectively, but not *C. liriodendri* isolates 1L, 2L and 3L (17.8, 18.8, 18.7 mm,

respectively) or *C. macrodidymum*, mean diameters for isolates 1M, 2M and 3M, being 14.7, 15.4 and 16.0 mm, respectively.

There was a significant treatment effect, with some differences between treatments (P≤0.05, LSD=1.50). Mycelium growth was most inhibited by chopped mustard plants + soil and least inhibited by chopped mustard plants without soil, mean diameters being 14.9 and 26.9 mm, respectively. The next most effective treatment was mustard meal, followed by mustard meal + soil with mean diameters of 15.7 and 17.5 mm, respectively. The mean diameters of mycelia in the controls were 21.9 and 22.1 mm, respectively, for MEA and soil controls.

The interaction between species and mustard treatments was significant (P≤0.001, LSD=3.42, Appendix F.2.3). The interaction appeared to be influenced by the overall resistance of *C. destructans* to mustard biofumigation treatments compared to *C. liriodendri* and *C. macrodidymum* which was most evident in Trt 3 (mustard meal), diameters being 24.5, 11.1 and 11.6 mm, respectively compared to controls 28.9, 19.6 and 16.9 mm, respectively (P<0.05, LSD=3.42). The chopped mustard plants + soil treatment was the most effective treatments against *C. destructans* species and overall as effective as mustard meal for inhibiting mycelium growth (17.0, 14.6 and 13.0 mm, respectively).

There was also a significant interaction between isolates and the mustard treatments (P≤0.001, LSD=4.49, Appendix F.2.1) particularly for the mustard meal (Trt 3) and chopped mustard plants + soil (Trt 2) in which mycelium growth (Figure 5.4) was significantly more inhibited for some isolates compared to their control. For example mycelium of *C. destructans* isolates 1D, 2D and 3D was significantly inhibited by Trt 2 compared to the MEA controls (21.5, 8.2 and 21.4 mm, respectively and 31.2, 23.6 and 32.3 mm, respectively). Mycelium growth of isolates 1D and 2D were significantly inhibited by Trt 3 compared to controls but not isolate 2D (Figure 5.4). For all three *Cylindrocarpon* spp. mycelium growth for two of the three isolates was significantly inhibited by mustard meal compared to the MEA controls. For example *C. destructans* 1D and 3D, *C. liriodendri* 1L and 3L and *C. macrodidymum* 1M and 3M (Figure 5.4) were significantly inhibited while isolate 2 of each species was similar to the control. The chopped mustard plants (Trt 1), was the least effective treatment overall; with this treatment mycelium growth for isolates of *C.*

destructans 3D, *C. liriodendri* 1L and 2L and *C. macrodidymum* 1M was significantly greater than the MEA controls (Figure 5.4).

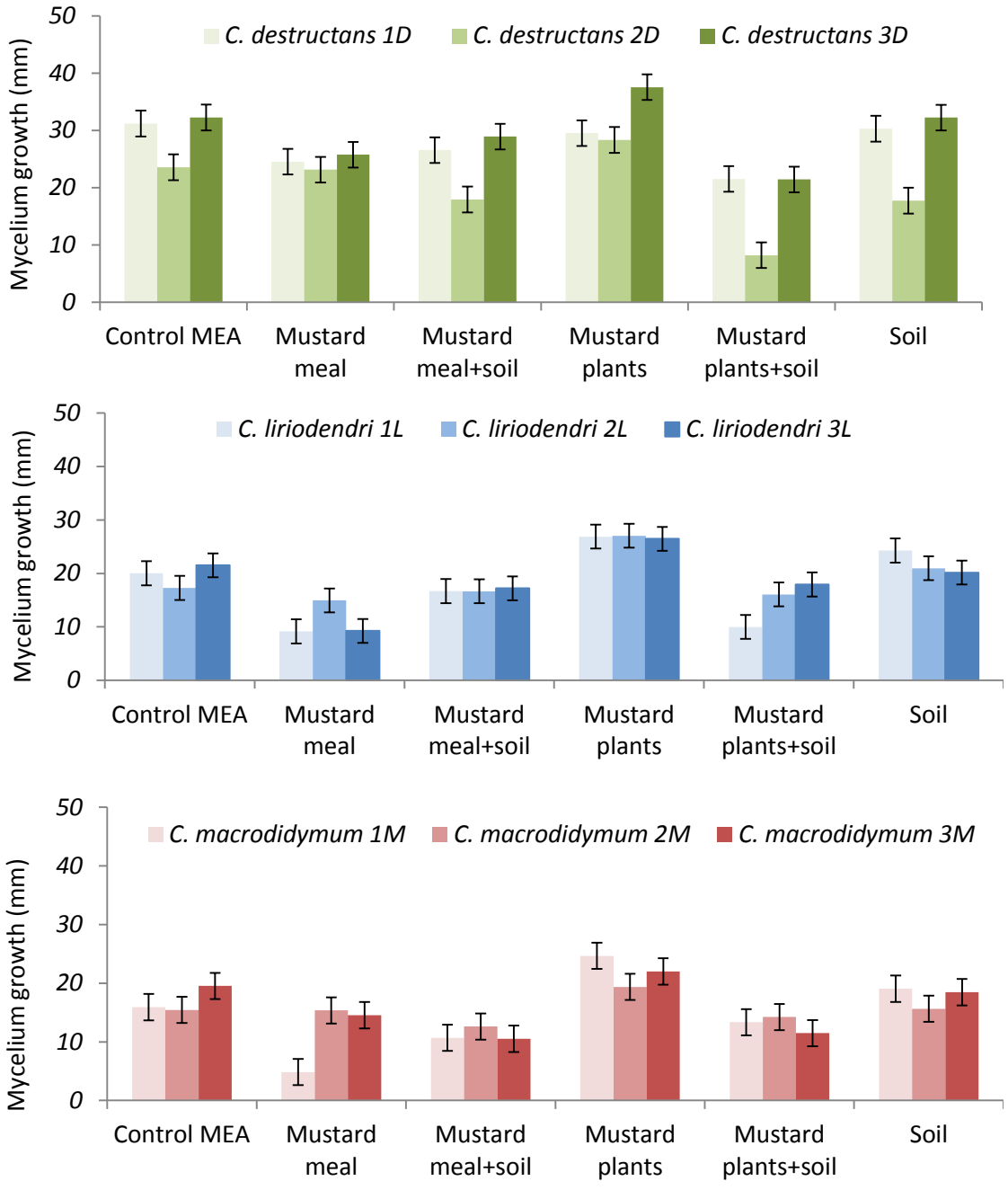


Figure 5.4 *Cylindrocarpon* isolate and treatment interaction for mycelium growth, with nine *Cylindrocarpon* isolates after biofumigation treatments with mustard meal and chopped mustard plants (incorporated into soil or not) as well as untreated MEA and soil controls (P≤0.05, LSD bar = 4.49).

5.7.2 Germination from conidial inoculum

There were significant effects of species (P≤0.001, Appendix F.2.2), isolates (P≤0.001, Appendix F.2.1) and treatments (P≤0.001, Appendix F.2.1) on development of colony forming units (CFU) from conidial inoculum. Mean CFU were less for *C. macrodidymum* than for *C. liriodendri* and for *C. destructans* (13.6, 20.6 and 29.7 CFU, respectively, P≤0.05, LSD 3.35). There was also variation in colony numbers across the isolates (LSD=2.98) of the species relative to the controls (number of CFU). For *C. macrodidymum*, isolates 1M, 2M and 3M mean number CFUs, were 20.2, 12.1 and 8.7, respectively, for *C. liriodendri* 1L, 2L and 3L mean number CFUs were 17.8, 27.4 and 16.8, respectively and *C. destructans* isolates 1D, 2D and 3D mean number CFUs were 46.5, 11.9 and 30.7, respectively.

There was a significant treatment effect with differences between all mustard treatments (P≤0.05, LSD=2.44, Appendix F.3.1). Colony forming units were significantly fewer, overall mean number CFUs (relative to the controls) being 4.1 after treatment with mustard meal (Trt 3) but when soil was added to the mustard meal (Trt 4) colony numbers were similar to the MEA control (both 24.1 CFU). All other treatments were similar to the MEA or soil control (24.1 and 25.5 CFU, respectively).

The significant interactions between species and treatment (P≤0.001, Appendix F.3.3) resulted from the different efficacies of the treatments to inhibit germination of the three species. After being exposed to mustard meal (Trt 3), mean number CFUs for *C. destructans* *C. liriodendri* and *C. macrodidymum* were 4.9, 4.9 and 2.6, respectively (LSD=8.2, P≤0.05), compared to chopped mustard plants (36.4, 30.3 and 16.5, respectively), mustard meal + soil (39.7, 20.5 and 11.7, respectively) and MEA control 29, 24.7 and 18.7, respectively; Figure 5.5).

The interaction between isolate and treatment (P≤0.001, Appendix F.3.1) was likely the result of some treatments causing greater inhibition of germination for some isolates within the species (Figure 5.5). For example when the mustard meal was mixed with soil (Trt 4) the average number CFU for *C. macrodidymum* isolates 1M, 2M and 3M were 22.8, 8.2 and 5.2, respectively which were more than for non-soil mustard meal treatments (Trt 3) 3.0, 4.8 and zero, respectively, and less than for soil controls (27.6, 20.0 and 13.6 CFU, respectively). However in the latter comparison, CFUs were not significantly different for isolate 1M (LSD=7.31, P≤0.05), (Figure 5.5). The mustard meal treatment also inhibited germination to

different extents between three isolates of *C. destructans* 1D, 2D and 3D and *C. liriodendri* 1L, 2L and 3L (CFUs being 13.2, 0.6, 1.0 and 1.2, 10.6 and 3.0, respectively) compared to the MEA controls (42.6, 11.6, 32.8 and 25.2, 29.8 and 19.2, respectively), (Figure 5.5).

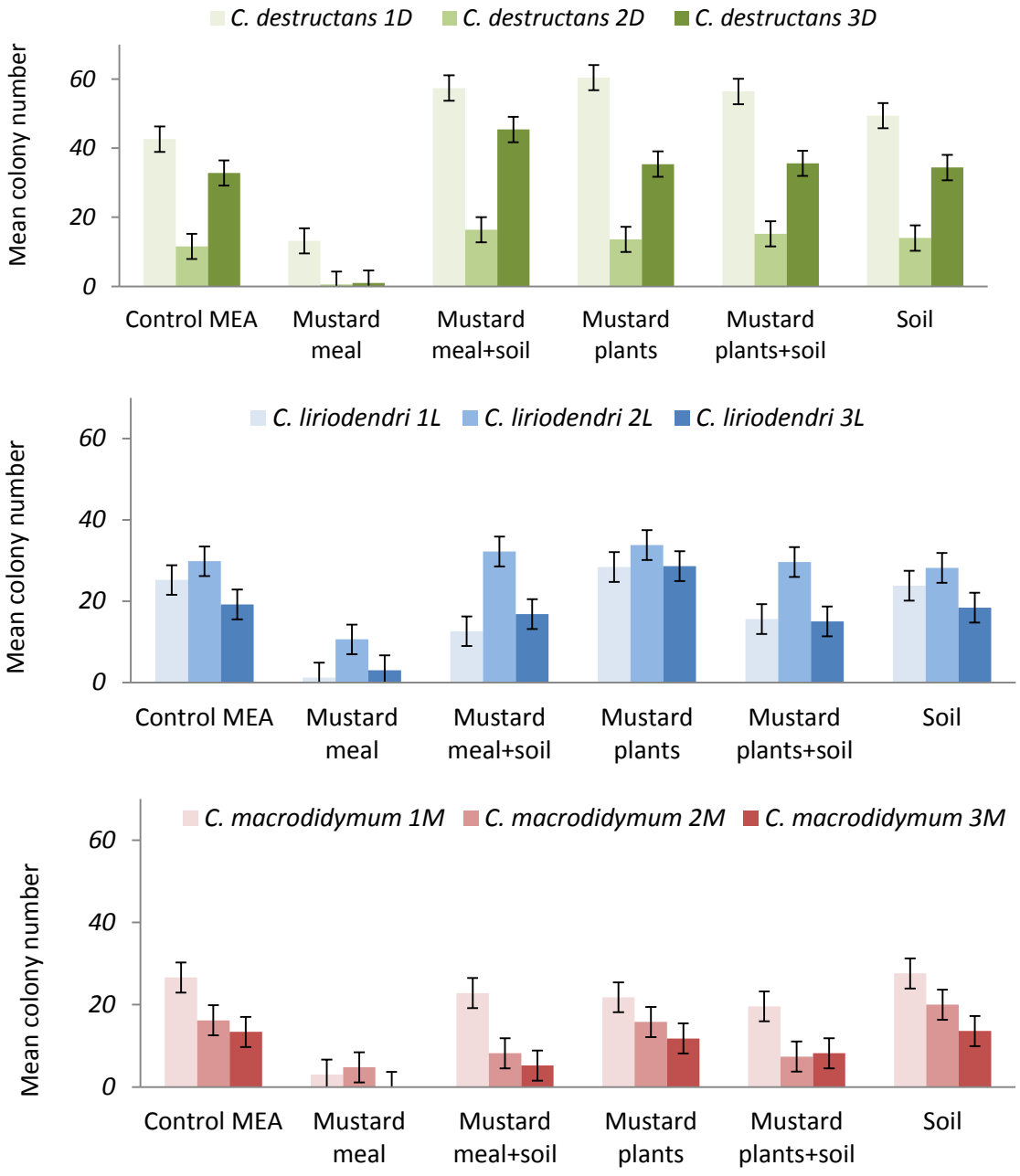


Figure 5.5 *Cylindrocarpon* isolate and treatment interaction for mean numbers of colony forming units (CFU) with nine *Cylindrocarpon* isolates after biofumigation using mustard meal and chopped mustard plants (incorporated into soil or not), as well as MEA and soil controls (P≤0.05, LSD bar =7.31).

5.7.3 Mycelial growth from conidial inoculum

There were significant effects of species (P≤0.001, Appendix F.4.2), isolates (P≤0.001, Appendix F.4.1) and treatments (P≤0.001, Appendix F.4.1) on colony sizes. Mean colony sizes were smallest for *C. macrodidymum* than for *C. destructans* and those of *C. liriodendri* were largest (4.1, 6.0 and 6.9 mm diameter, respectively, P≤0.05, LSD=0.46). There was also variation in colony sizes across the isolates (LSD=0.66) of the species. For *C. macrodidymum* isolates 1M, 2M and 3M, mean colony sizes were 4.3, 3.7 and 4.4 mm, respectively, for *C. destructans* isolates 1D, 2D and 3D, mean colony sizes were 5.0, 6.4 and 6.6 mm, respectively, and for *C. liriodendri* 1L, 2L and 3L, mean colony sizes were 6.4, 7.4 and 6.9 mm, respectively.

There was a significant treatment effect with differences between all mustard treatments (P≤0.05, LSD=0.54, Appendix F.4.1). Colonies were smallest after treatment with mustard meal (Trt 3) but not so restricted in size when soil was added to the mustard meal (Trt 4), mean colony sizes being 1.2, and 3.4 mm, respectively. The second most effective treatment was the chopped mustard plants (Trt 1), with the chopped mustard plants + soil (Trt 2) having significantly larger mean colony sizes, being 2.0 and 6.3 mm, respectively, whereas the controls had the greatest mean colony sizes, being 9.8 mm for MEA (Trt 5) and 11.4 mm for soil (Trt 6).

The significant interactions between species and treatment (P≤0.001, Appendix F.4.3) and isolate and treatment (P≤0.001, Appendix F.4.1) resulted from the greater efficacy of some treatments to inhibit colony growth of some species and isolates within species. For example the average colony size after *C. destructans*, *C. macrodidymum* and *C. liriodendri* spp. were exposed to mustard meal (Trt 3) were 0.7, 0.9 and 1.2 mm (LSD=1.12, P≤0.05) and chopped mustard plants 2.2, 1.4 and 2.2 mm, respectively compared to mustard meal + soil (3.8, 1.3 and 5.0 mm, respectively) and chopped mustard plants + soil (7.2, 3.3 and 8.3 mm, respectively). Even so colony size of all species was significantly reduced by the above treatments compared to the soil controls (11.5, 10.0 and 12.7 mm, respectively, P≤0.05).

The isolate x treatment interaction was associated with some treatments and isolates (Figure 5.6). For example, when the mustard meal was mixed with soil the colonies were often larger than the non-soil mustard meal treatments, but still significantly smaller than for controls (Figure 5.6). These effects differed between isolates, for example average colony

size for *C. liriodendri* isolates 1L, 2L and 3L exposed to mustard meal and mustard meal + soil were 0.4, 3.7 and 1.7 mm, respectively and 2.4, 6.8 and 5.6 mm, respectively compared to MEA control (11.3, 10.6 and 11.2 mm, respectively, LSD=1.61, P≤0.05). Overall, compared to their respective controls the biofumigation treatments significantly inhibited colony size for all isolates (Figure 5.6).

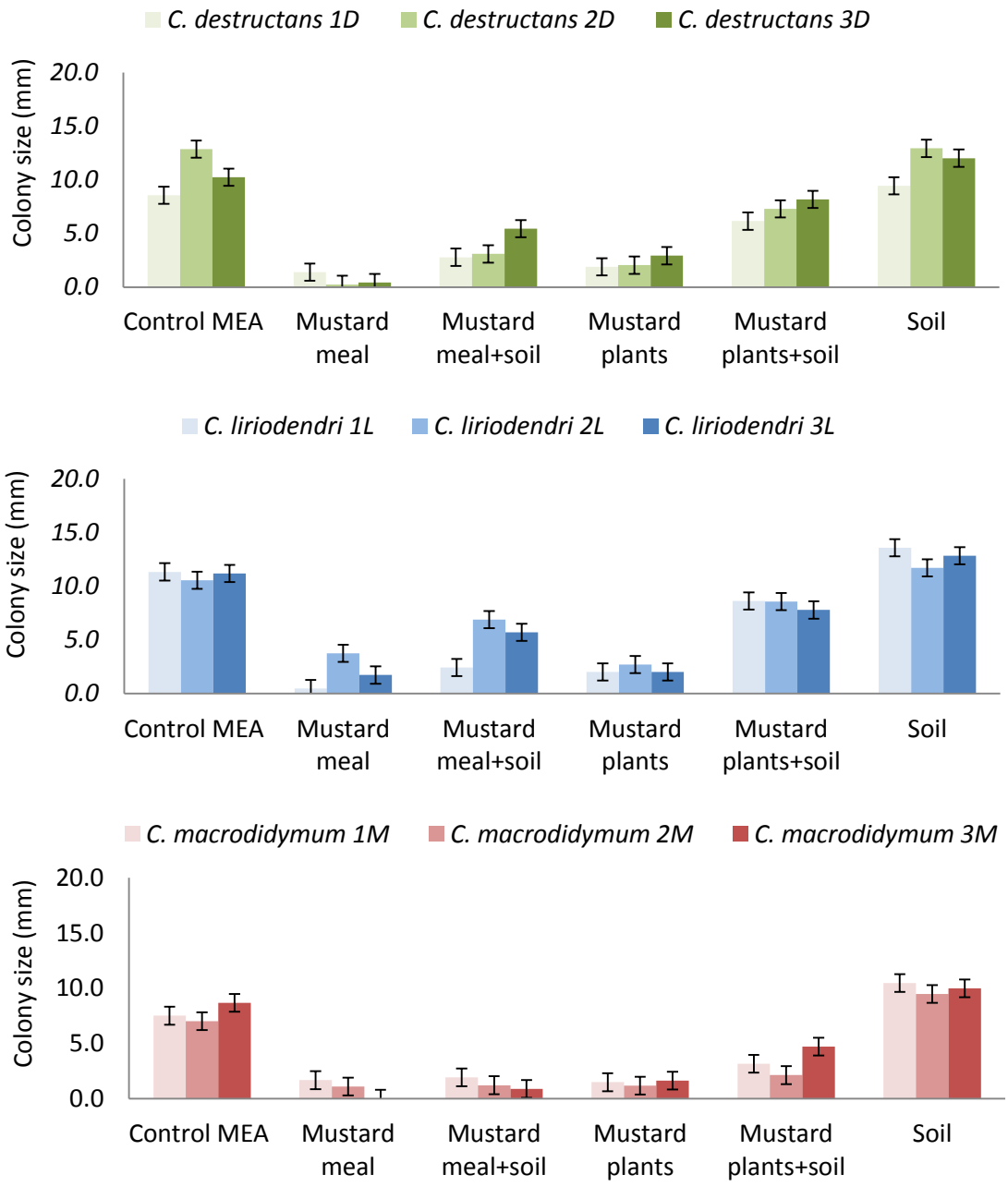


Figure 5.6 *Cylindrocarpon* isolate and treatment interaction for mean size of colonies that developed from conidia, for nine *Cylindrocarpon* isolates, after biofumigation using mustard meal and chopped mustard plants (incorporated into soil or not), as well as MEA and soil controls (P≤0.05, LSD bar = 1.61).

5.8 Discussion

The effects of chopped mustard plants and ground mustard seeds on mycelium growth, CFU and colony development from conidia of *Cylindrocarpon* isolates showed the potential for use of mustard biofumigation to control black foot disease in grapevines. The different effects observed between species and isolates was not unexpected since earlier *in vitro* experiments showed differences between species and isolates for fungicide effects (Chapter 2) and HWT effects (Chapter 3). Smith and Kirkegaard (2002) also reported that species and isolates differed in their responses to the biofumigation effect of 2-phenylethyl ITC (2-PE ITC), the main ITC released from the roots of canola, in a study that tested mycelium growth of 75 isolates from a range of fungi which included *Fusarium*, *Pythium*, *Rhizoctonia*, *Sclerotinia* and *Trichoderma* species .

In *in vitro* assays which used similar experimental methods as the current research, Larkin and Griffin (2007) reported that volatiles released from chopped Indian mustard inhibited growth of soilborne pathogens of potato: *F. oxysporum* and *Fusarium sambucinam* (Fr.) Sacc, (73 and 80 %, respectively), *Sclerotinia sclerotiorum* (Lib.) de Bary (90%) and completely inhibited *R. solani, Phytophthora erythroseptica* Pethybr. and *Pythium* spp. The chopped mustard plant material (1 g) was placed in the lid of a deep Petri dish taped to an inverted agar culture and the mycelium growth measurements were recorded after 24, 48 and 72 h (Larkin and Griffin, 2007). The fungal growth was similar for all three times which shows that the inhibition effect had occurred within the first 24 h and indicates the importance of immediate use of crushed mustard material to gain the greatest biofumigant effect. This concurs with Mazzola *et al*. (2007) who reported control of Rhizoctonia root rot of apple when *B. juncea* cv. Pacific gold and *R. solani* were introduced simultaneously into soil but no control when the pathogen was introduced 24 h after the mustard meal. The lethal effect of mustard material was demonstrated by Sammonds *et al*. (2012, Unpublished work) for mycelium and conidia of *Cylindrocarpon* isolates which were exposed to the biofumigant for 11 days in Petri dishes. The mustard material was then removed and the plates resealed, but after seven days there was no evidence of growth of the *Cylindrocarpon* isolates which indicated that the initial 11 day exposure was fatal and concurred with the findings of Larkin and Griffin (2007).

Morra and Kirkegaard (2002) also reported that the initially high concentrations of ITCs and GLS hydrolysis products produced by rapeseed and Indian mustard were prone to rapid degradation in soil. This was further investigated by Gimsing and Kirkegaard (2006) who reported that the concentration of both GSLs and ITCs in soil was highest immediately (30 min) after incorporation and could not be detected after 1-2 weeks. The current research showed that conidia that were not lethally affected in the first 24 h by the biofumigation treatments, germinated to produce colonies. However, those colonies were significantly smaller than the control colonies which indicated an enduring fungi-toxic effect for the 5 days prior to measuring colony sizes which was not sufficient to completely inhibit growth.

The *in vitro* study carried out at Lincoln University by Sammonds *et al*. (2012, Unpublished work) which used similar methods and materials to this study, tested the biofumigation efficacy of two mustard cultivars against *C. destructans*, *C. liriodendri* and *C. macrodidymum* isolates. Chopped plants and ground seeds of *B. juncea* and a new cultivar, Caliente 199, bred to have increased levels of both glucosinolates and the myrosinase enzyme (Seed and Field Services Ltd, 2009 in Sammonds *et al*., 2012), were equally effective for inhibiting mycelium growth of the three *Cylindrocarpon* spp. Germination of macroconidia of the three *Cylindrocarpon* species was completely inhibited by Caliente 199 and was low for *B. juncea,* being 0.0, 0.11 and 0.33%, respectively, for *C. liriodendri, C. macrodidymum* and *C. destructans*. This result agreed with the current study in which CFU and mycelium growth of *C. destructans* were overall least sensitive to mustard biofumigation.

Variation in the efficacy of different mustard cultivars was also reported by Montfort *et al.* (2011) from an *in vitro* study that investigated the effects of two strains of mustard for mycelium inhibition of *R. solani*. A Petri dish with chopped up *B. juncea*, strain B-1420 (4 g) inverted over a 500 mL jar containing the pathogen totally inhibited mycelium growth whereas strain A-Xceed 8571 (10 g) inhibited growth by only 20 to 30%. Montfort *et al*. (2011) hypothesized that the greater efficacy resulted from the plants' abilities to produce the glucoside 'sinigrin' (2-propenyl-GSL), which was significantly greater in the aerial parts of strain B than strain A plants (25.39 and 0.33 µmol g^{-1}, respectively). Unsurprisingly their findings showed that strain B had twice as much sinigrin in the aerial parts of flowering mustard plants and three times more in the roots than strain A (Montfort *et al.*, 2011), which most likely accounted for the greater inhibitory effect of strain B.

In the current research, mycelium growth, CFU and colony development from conidia of *C. macrodidymum* isolates were most affected by the mustard treatments whereas for *C. destructans* isolates mycelium growth and CFU were generally least affected, although isolate 2D was more sensitive to the treatments than isolates 1D and 3D (Figure 5.4 and 5.5, respectively). However, the chopped mustard plant treatment (Trt 1) was ineffective at inhibiting mycelium growth for all isolates except 1D. The time delay in macerating the mustard plants and placing them in the Petri dish may have resulted in a loss of ITCs and attributed to the ineffectiveness of this treatment. There was little difference in size of colonies that grew from conidia of *C. destructans, C. macrodidymum* and *C. liriodendri* but they were significantly smaller in size than their controls. These colonies differed from colonies grown from mycelium plugs since for some mustard treatments mycelium growth was greater than that of the controls. It clearly indicated that mycelium was less sensitive to the ITCs released from the crushed mustard plants and seeds than conidia. The reason was not clear but may have been caused by the increased sensitivity of germ tube development to brassicaceous secondary metabolites compared to spore germination or mycelial radial growth sensitivity (Sellam *et al*., 2007).

In choosing *Brassica* species with improved and high glucosinolate concentrations, the detectable period in soil may be extended (Morra and Kirkegaard, 2002). In addition, finding ways to best utilise the plant material or seeds and improve their incorporation into soil could further maximise ITC release and maintain its activity (Gimsing and Kirkegaard, 2006), and so could be further investigated in field trials similar to those carried out in this research.

Concern has been expressed by scientists (Rosa and Rodrigues, 1999) that biofumigation may negatively affect microbial antagonistic communities and in particular antagonistic *Trichoderma* spp. (Galletti *et al.*, 2008). Under controlled conditions Montfort *et al*. (2011) reported that *Trichoderma atroviride* was relatively unaffected by mustard biofumigation, which concurred with Galletti *et al*. (2008) who reported that in an *in vitro* assay *Brassica carinata* seed meal combined with *Trichoderma* spp. improved mycelium inhibition of *Pythium ultimum* Trow, and suggested that in combination it could be used as an integrated biocontrol of soil pathogens. In soil under controlled conditions *Trichoderma* spp. showed promise for reducing *P. ultimum* disease incidence of sugar beet however, Galletti *et al*.

(2008) recommended further investigation was required *in vitro* and under field conditions using different mustard varieties. Chapter 2 of this research showed that some plants treated with Tricho-Flow™ had reduced *Cylindrocarpon* infection, therefore an integrated control for black foot disease using *Brassica* and *Trichoderma* species may be beneficial and could be investigated under field conditions.

The current study showed differences in the efficacy of the treatments to inhibit CFU germination, development of conidia into colonies and mycelium growth. It also showed that conidia were more susceptible to the raw material of mustard meal (Trt 3) and chopped mustard plants (Trt 1), respectively than mycelium which was inhibited most by the chopped mustard plants + soil (Trt 2) and mustard meal (Trt 3), respectively and least inhibited by the chopped mustard plants (Trt 1). Since the mustard meal was lightly sprayed with SDW in the *in vitro* conidium experiment, but not in the mycelium assays, this may have caused the greater efficacy of that treatment to inhibit conidium development, since water facilitates myrosinase enzymatic hydrolysis of glucosinolates, which are relatively inactive until hydrolysed to ITCs (Kirkegaard and Sarwar, 1998). That would have occurred naturally in treatments where the raw material was added to soil since it was at 80% field capacity and also during the process of blending the mustard plants with small amounts of SDW to avoid clumping.

Montfort *et al.* (2011) reported that biofumigation effects in soil were not always consistent which agreed with the current findings, since addition of soil sometimes made the mustard material less effective against conidia as the average colony sizes were much larger for treatments of chopped whole mustard plants + soil (Trt 2) and mustard meal + soil (Trt 4) than the same treatments without soil, for example with isolates of *C. destructans* 3D and *C. liriodendri* 2L and 3L, although colonies were still significantly smaller than for controls. Different soil types may affect brassica biofumigant efficacy as was reported by Mazzola *et al.* (2001). The addition of soil to the whole mustard plants increased the efficacy of that treatment. Soil microbes may have influenced the result although this was not tested. This result indicates that further research with different soil types and isolates is justified. Stephens *et al*. (1999) reported that chopped Indian mustard roots and shoots inhibited growth of an isolate of *C. destructans* on agar in an *in vitro* experiment but not when the mustard material was mixed with soil under field conditions and so suggested that field

experiments gave a more accurate indication of the efficacy of brassica inhibition than *in vitro* studies. Regardless, the *in vitro* assays in this research provided good insights into the various effects of the biofumigation treatments against *Cylindrocarpon* species and isolates within the species, as well as their effects on development of mycelia and conidia.

The positive results of the *in vitro* assays justified further investigation and, since little research had been reported for the control of black foot disease of grapevines using mustard biofumigation, an experiment was conducted under field conditions.

5.9 Section 2: - *In vivo* mustard biofumigation experiments

5.10 Material and methods

In October 2008, a biofumigation experiment was set up at a Lincoln University site (HRA) near the site of the preliminary experiment (Section 5.3) to test the efficacy of crops of mustard plants and mustard meal incorporated into the soil to control infection by *Cylindrocarpon* spp. in rootstock varieties 101-14 and 5C prepared as described previously (Section 2.8.3).

5.10.1 *Cylindrocarpon* spp. inoculum

Mycelium of the nine *Cylindrocarpon* isolates of *C. destructans* (1D, 2D and 3D), *C. liriodendri* (1L, 2L and 3L) and *C. macrodidymum* (1M, 2M and 3M) was produced on sterilised wheat grains. The wheat grains (222 g) were placed in a 1L conical flask containing 500 mL of tap water and 62.5 mg of chloramphenicol and heated to boiling. The grains were left to settle for 10 min then washed three times with tap water and the excess drained off. The grains were autoclaved at 121°C for 15 min at 15 psi and left for 24 h, after which the process was repeated. Each flask of wheat grains was inoculated with five 6 mm mycelium plugs of one isolate taken from the edge of the appropriate *Cylindrocarpon* cultures grown on PDA plates and incubated at 20°C for 2-4 weeks. The flasks were incubated at 20°C in the dark for 14 days, during which they were shaken daily by hand (5 sec) to assist colonisation by mycelium which was confirmed visually (Figure 5.7). The contents of the flasks were thoroughly mixed in a clean 20 L plastic bucket prior to inoculation of the field site.

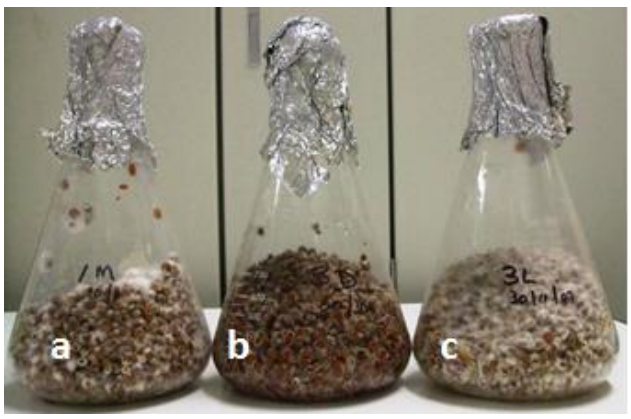


Figure 5.7 Conical flasks containing wheat grain inoculated with *Cylindrocarpon* isolates 1M (a), 3D (b) and 3L (c).

5.10.2 Treatments

The mustard treatments used were: mustard meal (Trt 1), mustard grown once to flowering (50% anthesis) then cultivated into the soil (Trt 2), mustard grown twice to flowering and cultivated into the soil (Trt 3), and bare land control (Trt 4). The field site was rotary hoed (October 2008) and the surface raked to give a fine tilth. The plots assigned to Trt 3, (two mustard crops) were immediately sown with *B. juncea* mustard seed (15 g/m^2), lightly raked to cover seed with soil and watered as described in Section 5.3.2. After four weeks, when the plants had reached 50% anthesis all plots were again rotary hoed and the chopped mustard plants (Trt 3) incorporated into the soil of those plots and the site irrigated by sprinkler (30 min) as described in Section 5.3.2. The following day the soil was lightly raked and mustard seeds (15 g/m^2) sown into the plots allocated to Trt 2 and Trt 3, as described above. After six weeks when mustard plants had reached 50% anthesis, the site was rotary hoed and the chopped mustard plants incorporated into the soil. At the same time, the mustard seed for treatment one was finely ground in a mortar and pestle and the mustard meal (80 g) mixed by hand with a garden fork into the top 20 cm of each plot allocated to that treatment. At the same time all plots were infested with the colonised wheat grain (110 g/m^2) described above (Section 5.9.1) which was mixed by hand through the top 20 cm of soil which contained the mustard material or bare soil. The site was immediately covered with black polythene and irrigated for 30 min by T-Tape which had been laid beneath the polythene, as described in Chapter 2.

5.10.3 Field site preparation

After two weeks, cuttings of the two callused rootstock varieties 101-14 and 5C were planted through the polythene into the treated soil and maintained as described previously (Section 5.3.3).

5.10.4 Assessment

The plants were grown for 10 months, then harvested and infection assessed as described previously (Section 2.9.1).

5.10.5 Statistical Analysis

The data of *Cylindrocarpon* spp. mean disease incidence per plot (from 0 cm and 5 cm isolations) and severity (the mean proportion per plot of tissues infected at 0 cm) were

analysed by general linear model (GenStat Release 14.1, VSN International Ltd, U.K.) as described in Section 5.3.5.

5.11 Results

There was a non-significant trend towards a reduction in disease incidence and severity in plants at 0 cm or 5 cm or severity (P=0.359, P=0.785 and P=0.453, Figure 5.9), (Appendix F.5.2, F.5.3 and F.5.1, respectively). There were significant differences between rootstock varieties for disease severity and incidence (at 0 cm and 5 cm) in plants (all P≤0.001, Appendix F.5.1, F.5.2 and F.5.3, respectively). Disease incidence (0 and 5 cm) and severity was greater in rootstock variety 101-14 than 5C (60 and 34 and 40%, respectively and 29, 13 and 11%, respectively LSD=9.41, 10.26 and 7.32, respectively, P≤0.05), (Figure 5.8).

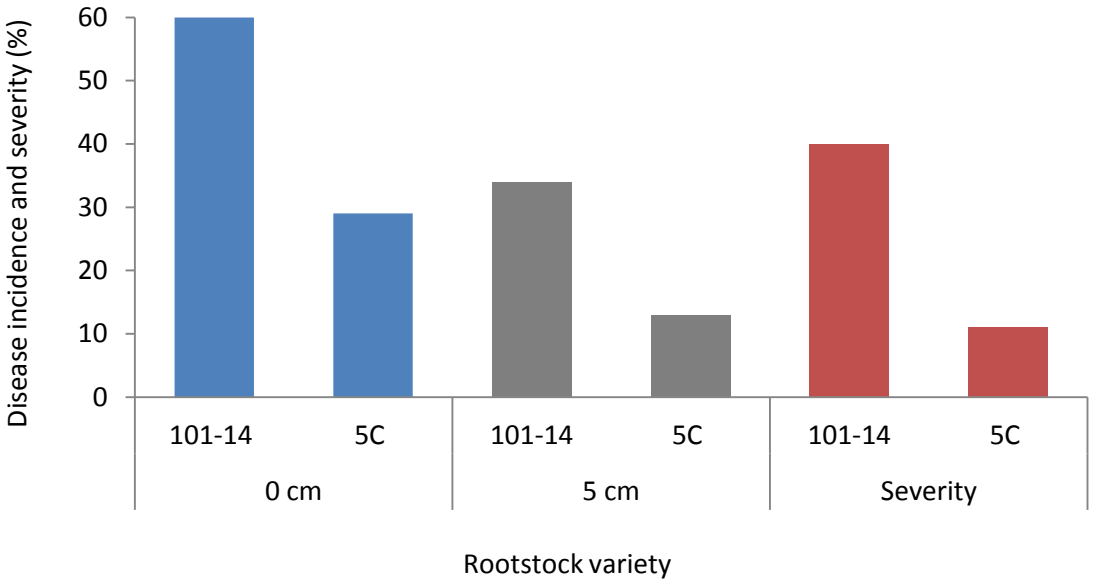


Figure 5.8 Mean percent incidence at 0 cm (P<0.001, LSD=9.41) and 5 cm (P<0.001, LSD=10.26) and severity (P<0.001, LSD=7.32) of *Cylindrocarpon* spp. in rootstock varieties 101-14 and 5C planted into soil amended with mustard meal, mustard sown once, mustard sown twice and an untreated control (Fishers protected LSD P<0.05).

The treatments were not significant however incidence (0 cm and 5 cm) and severity was reduced by mustard meal and mustard sown twice but not mustard sown once only (Figure 5.9).

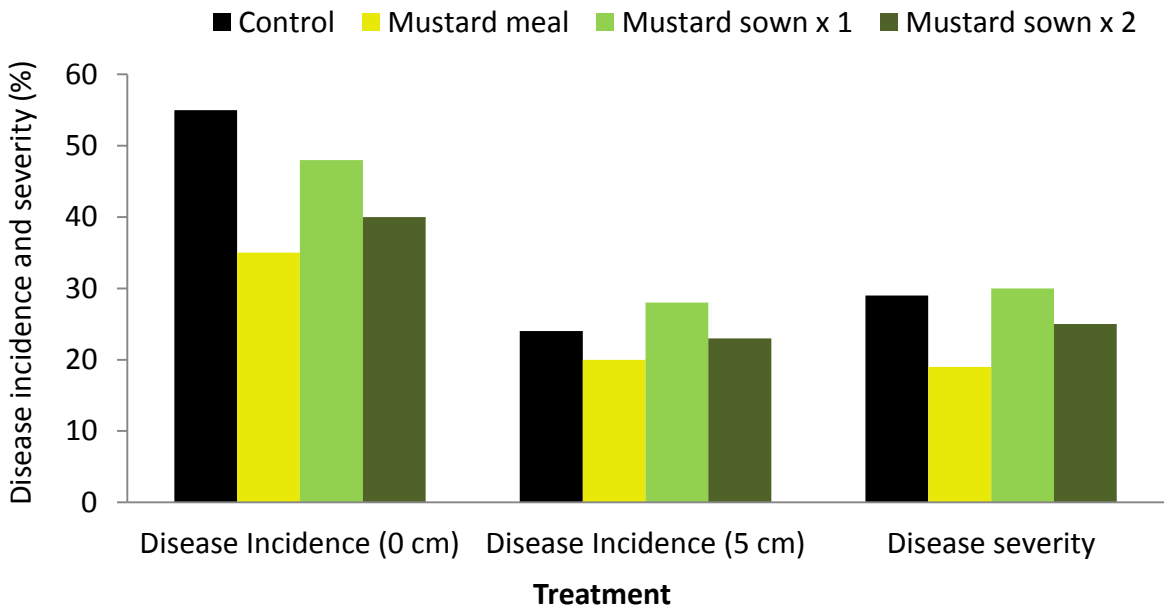


Figure 5.9 Mean percent incidence at 0 cm (P=0.359) and 5 cm (P=0.784) and severity (P=0.453) of *Cylindrocarpon* spp. in grapevine plants of variety 101-14 and 5C planted into soil amended with mustard meal, mustard sown once, mustard sown twice and an untreated control (Fishers protected LSD P>0.05).

Treatment results for incidence (0 cm and 5 cm) and severity, as percentage reductions of the effects in control plants were: mustard meal (36, 17, and 34%, respectively, P≥0.05), mustard sown twice (27 and 4 and 14% respectively, P≥0.05) and mustard sown once (13, -17 and -3%, P≥0.05). There were no significant treatment and variety interactions for disease incidence (0 and 5 cm) and severity in plants (P=0.193, P=0.365 and P=0.481, respectively).

When the data was analysed using the Dunnett t-test, with a P value of P<0.10 which may be considered appropriate from a biological perspective, there was a significant treatment effect for disease severity (P=0.095, Figure 5.10) (Appendix F.5.4) but not disease incidence at 0 and 5 cm (P=0.190 and P=0.426, respectively). Disease severity for plants of the mustard meal and mustard sown twice treatments were similar (19 and 25%, respectively LSD=7.50) but disease severity for plants treated with mustard meal was significantly less than for plants of mustard sown once and the control treatments (30 and 29%, respectively) which were similar to plants of the mustard sown twice treatment (Figure 5.10).

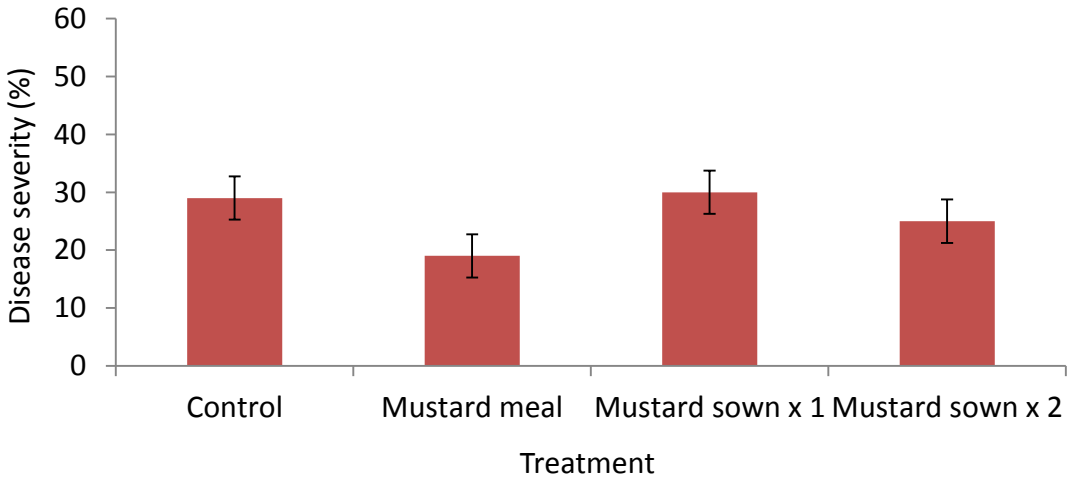


Figure 5.10 Mean percent disease severity (P=0.095, LSD=7.50) of *Cylindrocarpon* spp. in grapevine plants of variety 101-14 and 5C planted into soil amended with mustard meal, mustard sown once, mustard sown twice and an untreated control (Dunetts P-value, P<0.10).

5.12 Discussion

The results of this research showed that biofumigation with mustard plants and ground seeds had some potential for improving control of black foot disease in grapevines. Disease severity in plants that were planted in soil that had been treated with mustard meal was significantly reduced compared to control plants (P<0.10). Overall, under field conditions grapevine plants from the mustard meal and twice-sown mustard (Trt 1 and Trt 3) had reduced disease incidence by 36 and 27%, respectively. Since soil-borne inoculum of this pathogen is difficult and/or expensive to eradicate by fumigation treatments, mustard may offer a real solution to nursery propagators or vineyard owners who have some spreading patches of vines with this disease.

Biofumigation with *Brassica* species has been actively studied over the last 50 years (Kirkegaard *et al.*, 1993; Matthiessen and Kirkegaard, 2006; Montfort *et al.*, 2011; Rosa and Rodrigues, 1999). These research efforts have provided evidence of the long recognised allelopathic effects which may be used for pest management in agricultural systems (Brown and Morra, 1997; Rosa and Rodrigues, 1999). The efficacy of *B. juncea* species to control multiple soilborne pathogens has been reported under field conditions on crops of potatoes (Larkin and Griffin, 2007) and support the findings of this research. On two commercial

potato farms in north and central Aroostook County, Australia, both with a history of soilborne diseases, Larkin and Griffin (2007) conducted field trials with crops of rapeseed, yellow mustard, Indian mustard, canola and rotation crops of ryegrass and oats to measure their ability to reduce inoculum of soilborne pathogens and disease development by *R. solani* (black scurf), *Streptomyces scabiei* (Thaxter) Lambert & Loria (common scab) and *Spongospora subterranean* (Wallr.) Lagerh. F. sp. *subterranean* Tomlinson (powdery scab). Crops were sown at both sites, excluding canola at the central Aroostook County site, and after 60-70 days they were ploughed into the soil as green manure. The following spring 'seed-potato pieces' were planted, and after four months the percent disease incidence (numbers of tubers showing substantial disease of 2% or greater surface coverage) and severity (average percent of tuber surface covered with symptoms) was determined (Larkin and Griffin, 2007). At the northern Aroostook County site all brassica crops reduced the severity of powdery scab compared to oats (25-38% reduction), of which Indian mustard was the most effective, reducing incidence by 38%. However black scurf incidence and severity was even further reduced by rapeseed and canola (71 and 78%, respectively) but Indian mustard was ineffective against black scurf as mean disease incidence and severity was 9% greater than in the oats control. In the central site where common scab was predominant (95%) Indian mustard provided the greatest level of control, reducing disease incidence and severity by 12-25% and 12-22%, respectively, relative to crops of ryegrass, yellow mustard and rapeseed. Overall, Indian mustard which contained the highest GLS concentrations (Larkin and Griffin, 2007) was the most effective *Brassica* crop and is comparable to the control achieved in the current research with *B. juncea* sown twice.

Noteworthy, in the field experiment carried out by Larkin and Griffin (2007) was that ploughing the site provided enough damage and incorporation of the *Brassica* crops to initiate GSL hydrolyses and reduced levels of soil borne pathogens. In the current study, rotary hoeing the mustard plant material into the soil seemingly caused enough damage to activate GLS hydrolyses. In contrast Gimsing and Kirkegaard (2006) proposed that ploughing could be an inhibitory factor for the biofumigation process under field conditions if thorough pulverisation of the plant tissues was not achieved during the incorporation process. The glucosinolates found in *Brassica* species are not biologically active until they are enzymatically hydrolysed by the degradative myrosinase enzymes to ITCs, nitriles, thiocyanates, and oxazolidinethiones (Bones and Rossiter, 1996). Consequently the damage

to plant cells in the presence of water is an integral mechanism to the biofumigation process which was adhered to in the current research by immediate irrigation of the site after incorporation of the mustard treatments. A key to improving the efficacy of biofumigation in the field lies in the development of application technologies that macerate and incorporate the biofumigant evenly in soils, as well as incorporating it under optimal edaphic conditions for release of ITCs (Mattner *et al.*, 2008). Additionally, the effectiveness of disease suppression using biofumigation and green manure plants requires that they are not host to the target pathogen (Matthiessen and Kirkegaard, 2006) which has not been investigated for *Cylindrocarpon* spp., however a search of the literature produced no reports of *C. destructans*, *C. liriodendri* or *C. macrodidymum* on *Brassica* species.

Smolinska (2000) reported that the toxic compounds released from decomposition of *B. juncea* and *B. napus* predisposed the sclerotia of *S. cepivorum* to parasitism by saprophytic microflora. Sclerotia were recovered from soil treated with *B. napus* cv. Gorczanski and *B. napus* cv. Bolko plant materials, and the 'majority' of those treated with *B. juncea* were soft and non-viable whereas those from untreated soils remained hard and viable. However, for *F. oxysporum* chlamydospores, only *B. juncea* was effective, with percentages of viable propagules after 25 and 60 days being significantly reduced. Chlamydospores added to the brassica amended soil were reduced to 65 and 13%, and chlamydospores from the suspension of *Fusarium* conidia added to soil were reduced to 5 and 14%, respectively, compared to controls (100%). *Fusarium* chlamydospores are sensitive to isothiocyanates (Smolinska *et al.*, 2003) and since *B. juncea* contains GLS that release high concentrations of ITCs (Rangkadilok *et al.*, 2002; Smolinska *et al.*, 2003) its deadly effect to *F. oxysporum* chlamydospores was not unexpected. Since previous research by Probst (2011) reported that mycelium and conidia of *C. liriodendri*, *C. destructans* and *C. macrodidymum* species were converted to chlamydospores in soil, the capacity of *B. juncea* species to lethally damage chlamydospores may be very effective in removal of surviving soil-borne inoculum. The effect of biofumigation on *Cylindrocarpon* chlamydospore survival could be investigated using the soil assays of Probst (2011).

In Australia, biofumigation with *Brassica* species was tested in grapevine nurseries to find alternative fumigation treatments to methyl bromide (MeBr) and metham sodium which have been routinely used to control root pathogens of Shiraz on Ramsey rootstocks

(Stephens *et al.*, 1999). In that study, Indian mustard crops had a negative effect on grapevine establishment compared to control plants (87.5 and 93.5%, respectively) and did not reduce infection by *Cylindrocarpon* spp. or *Rhizoctonia* spp. Therefore the incorporation of Indian mustard compared to the chemical fumigants was not considered effective or economically beneficial (Stephens *et al.*, 1999). However, since 2005 the use of ozone depleting chemicals such as methyl bromide has been phased out and so they are no longer available. While the control effects were not encouraging, Stephens *et al.* (1999) also reported a significant increase in the number of *Pythium* propagules in the soil in plots treated with Indian mustard and canola and suggested that Pythium damping-off may have played a greater part in reduced grapevine take than the mustard treatment. Indeed the incorporation of *Brassica* crops have been reported to stimulate saprophytically growing soil bacteria and fungi by providing increased amounts of growth substrate (Friberg *et al.*, 2009), a result that was also observed in pot trials by Smolinska (2000) but was not investigated in this study. One month after incorporation into the soil, both *Brassica* species had significantly increased numbers of bacteria and fungi (*Mucor hiemalis*) compared to untreated soil. However after two months the differences were not significant and after one year only bacterial numbers remained elevated (Smolinska, 2000).

Mazzola *et al*. (2007) reported that soil microflora may be positively influenced by using a combination of *B. juncea* and *B. napus* seed meal mixture, which may also improve control of the pathogen complex inciting apple replant disease. These findings were subsequent to the study by Mazzola *et al*.(2001) in which *Rhizoctonia* spp. were effectively reduced in roots of Gala apple seedlings grown for 12 weeks in pots containing apple replant soil amended with two concentrations (1.0 and 0.1% vol/vol) of *B. napus* 'Dwarf Essex' or canola seed meal. In contrast, the seed meal of *B. napus* was relatively ineffective against *Cylindrocarpon* spp., although Mazzola *et al*. (2001) reported some efficacy with a lower concentration in one soil type. Mazzola *et al.*, (2007) also reported that *B. juncea* seed meal did not increase populations of *Pythium* whereas *B. napus* did, which contrasted with the results reported by Stephens *et al.* (1999). The increased *Pythium* population corresponded with increased root infection of apple seedlings. Mazzola *et al*. (2007) also reported the greatest biofumigation effect was within the first 24 h after seed meal application and recommended an application rate of 8 to 10 t ha^{-1}. Although not tested, the apparent relationship between *B. napus* and

Pythium population expression may have accounted for the relative lack of efficacy of *B. napus* in the current study.

The *in vitro* experiments and to a lesser extent the field experiments reported in the current study provided strong evidence to support the efficacy of mustard biofumigation to control *Cylindrocarpon* spp. However further field research with more effective mustard varieties and more replication, so less variation, are required to support these findings and validate the usefulness of biofumigation as a control method. Ultimately, this could provide a sustainable control method against black foot disease in nurseries and vineyards.

Chapter 6

Concluding Discussion

This PhD thesis represents a body of research work which investigated methods for control of Cylindrocarpon black foot disease. The pathogens responsible for black foot in New Zealand were identified from grapevine samples gathered from all the major grape growing regions of New Zealand. The three species, *C. destructans* (*Ilyonectria radicicola* species complex), *C. liriodendri* and *C. macrodidymum* (*Ilyonectria macrodidyma* complex) were found in similar proportions in newly planted vineyards and nurseries, where the disease was particularly problematic. Since black foot was estimated to cause 10% loss of young vines (B. Corban pers. comm. 2007) in an industry that exported $1.18 billion of wine in 2011 (Smith, 2012), there was a clear economic need to develop control methods for nursery and vineyard use.

The investigation included experiments to determine the potential for management of black foot disease by chemical, cultural and biological strategies that were suitable for New Zealand's cool climate viticulture. This was carried out by testing *in vitro* the efficacy of fungicides, HWT and mustard biofumigation to mycelium and conidium viability of nine isolates, three each of the three *Cylindrocarpon* species found in New Zealand vineyard grapevines. Selected treatments were then evaluated in commercial nurseries in soil artificially infested with a mixed inoculum of *Cylindrocarpon* spp. before planting. This research is the first to test chemical and cultural control methods for their effects on mycelium growth and conidium viability for all three *Cylindrocarpon* species, *in vitro* and *in vivo*, and the first to investigate mustard biofumigation as a potential method of reducing *Cylindrocarpon* species viability in soils.

6.1 Disease management

6.1.1 Fungicides

In the *in vitro* fungicide experiments, the EC_{50} values indicated that only three of the 12 fungicides tested, captan, carbendazim and didecyldimethyl-ammonium chlorine, effectively inhibited both mycelium growth and conidium germination of all nine *Cylindrocarpon*

isolates (Chapter 2). Of the other fungicides tested, some fungicides inhibited mycelium growth but did not inhibit conidium germination and *vice versa*, a phenomenum also reported by overseas researchers (Chapter 2). However, the analysis by ANOVA showed that the fungicides, cyprodinil + fludioxonil, fludioxonil and tebuconazole were similarly effective and reduced both mycelium growth and conidium germination of most isolates, although *C. liriodendri* isolates, especially isolate 3L, showed greater resistance to the fungicides tested.

The results of the field experiments differed between the Auckland and Blenheim sites. Captan and carbendazim + flusilazole were the most effective chemical treatments in Auckland while in Blenheim those fungicides were relatively ineffective, with cyprodinil + fludioxonil and the biocontrol agent Tricho-Flow™ being most effective. In the Lincoln field site, cyprodinil + fludioxonil and Tricho-Flow™ effectively reduced the incidence and severity of *Cylindrocarpon* spp. This result concurred with overseas research which reported that in naturally infested field sites carbendazim + flusilazole and cyprodinil + fludioxonil significantly reduced disease incidence (Rego *et al.*, 2009).

The infestation of the experimental sites with the mixed *Cylindrocarpon* spp. inoculum proved to be unnecessary as the plants from non-inoculated treatments also had relatively high disease levels. Furthermore, the increased disease pressure in the inoculated sites may have caused an unusually great challenge for the fungicides, resulting in reduced efficacy for the pre-planting treatments. However the HWT (47°C for 30 min) seemed unaffected by the high soil inoculum concentration and was the most effective treatment against the black foot pathogens at both sites.

Based on the results of this research, grapevine preplant fungicide dips using captan, carbendazim, cyprodinil + fludioxonil (as Switch™), carbendazim + flusilazole and Tricho-Flow™ could be recommended. However as the use of carbendazim, including as post-harvest dips was banned in January 2010 in Australia (Stewart, 2012), the likely ban of carbendazim in New Zealand is imminent. Didecyldimethyl-ammonium chlorine while not generally believed to be suitable for a soil environment showed very good efficacy against *Cylindrocarpon* propagules and could be incorporated into nursery systems for use in post-harvest, propagation and grafting processes as a prophylactic treatment on grapevine material.

6.1.2 HWT

The HWT of 47°C for 30 min was most effective in reducing incidence of black foot pathogens in plants in the first field trial (Chapter 2), consequently further research investigated HWT protocols using different temperature and time combinations. The aim was to find a treatment that was fatal for the nine *Cylindrocarpon* isolates without jeopardising the health of grapevine material grown under New Zealand conditions. With increased HWT temperature and time, mycelium growth and conidium germination were progressively more inhibited. Conidium germination was more inhibited than mycelium growth; after HWT at 40, 45 and 47°C for 15 min conidium germination was completely inhibited, whereas the mycelium plugs required an increased HWT time of 30 min at 45, 47, 50 and 55°C to greatly inhibit mycelium growth. Isolates susceptibility to HWT varied between and within species, with mycelium of *C. liriodendri*, mainly isolate 3L, and conidia of *C. destructans* spp. being least susceptible to HWT. However, HWT for 30 min at 47 to 50°C was the optimum range and inhibited the mycelium growth of the more resistant isolates

A further *in vitro* experiment again tested the effects on the nine isolates which had been imbedded into grapevine canes then HWT for 30 min at 47, 48.5 or 50°C (Chapter 3). Results showed that *C. macrodidymum* isolates, which were very sensitive to the earlier HWT treatments, were not as sensitive when inside the canes as the other species to treatment at 47 and 48.5°C, although 50°C for 30 min completely eliminated this fungus from the grapevine canes. In contrast, *C. liriodendri* isolates, which were more heat tolerate in earlier experiments, were almost completely eliminated by treatment at 48.5 and 50°C. Regardless, this experiment showed that HWT using a temperature lower than the industry standard was effective against the nine New Zealand *Cylindrocarpon* isolates implanted into grapevine canes. This research showed that the modified HWT of 48.5°C for 30 min could replace the industry standard HWT, eliminating *Cylindrocarpon* spp. from trunks of grapevine plants which had become naturally infected while growing in soil infested with the black foot pathogens. The less stringent treatment was a useful innovation as it has the potential to decrease the reported losses of HWT plants grown in cool climates (Chapter 3).

The field trial of HWT effects on vineyard plants aimed to investigate the combined effects of variety, HWT, cold storage and harvesting date. It is the first investigation of the effects of these treatments and their interactions on bud development and carbohydrate

concentrations in field grown, young grafted grapevines. These treatments had been reported overseas to affect health of young vines after planting out, although these studies did not investigate the physiological effects on the vines. Overseas, HWT and cold storage protocols were reported to have caused physiological damage to cuttings and rooted vines although the mechanisms were not well understood (Chapters 3 and 4). The grapevines were planted in infected soil and the effects of the treatments examined. After they had been grown in infested soil, this research examined the effects of the treatments with respect to disease incidence and severity, subsequent plant growth and concentrations of water soluble carbohydrates in roots, including low and high molecular weight sugars and starches (Chapter 4).

Disease incidence and disease severity was significantly reduced by HWT but not by cold storage. Harvest month had an interesting but unexplainable effect since *Cylindrocarpon* spp. infection levels significantly reduced in the grafted grapevines during the harvest months, which also coincided with a reduction in root starch concentration (Chapter 4). Throughout this research, rootstock 101-14 generally had greater disease incidence and severity than 5C. In the grafted grapevines, infection also appeared to be influenced by the cultivar, since infection was significantly greater in 101-14 cv. Pinot noir than in 101-14 cv. Sauvignon blanc.

Root carbohydrate concentrations were affected by the treatments. Over the months of harvest sugar concentrations remained relatively constant whereas starch concentrations continually reduced. There was also a significant effect of cold-storage since much higher concentrations of sugar and lower concentrations of starch were measured in cold-stored than in non-cold stored plants. This seemed to indicate that starch was converted to sugar in cold stored plants but not in non-cold stored plants. Increased respiration during early stages of cold storage had been reported (Chapter 4) and possibly accounted for the significant overall reduction observed in root starch content of cold stored grapevines.

HWT cuttings had been reported to establish more slowly than non HWT cuttings but that hypothesis was not supported by this research. This research showed that HWT young grapevines developed earlier than non-HWT ones and that bud growth was quicker for plants that were cold stored than in non-cold stored plants. However, bud growth for plants that were harvested in July, the coldest month, was similar for all treatments. In contrast to

anecdotal reports that HWT affected the viability of HWT plant material (Chapter 3), no plants died during this experiment, validation for the use of the reduced HWT for grafted grapevines grown in cooler climates.

These results therefore indicated that grapevines or cuttings should be harvested when they are in deepest dormancy and if not they should be cold stored for a four week period prior to HWT and or normal spring growth. In warmer climates where grapevines do not undergo dormancy, cold storage could be used to reduce the time taken for plants to break bud and to ensure uniform development, as well as to potentially reduce disease incidence.

6.1.3 Biofumigation

The allelopathic effect of biofumigation using *Brassica* species and the efficacy of *B. juncea* to control multiple soil-borne pathogens was discussed in Chapter 5. This research demonstrated the efficacy of chopped mustard plants + soil and ground mustard seed for inhibiting mycelium growth, conidial germination and colony development from conidia of *Cylindrocarpon* isolates *in vitro*. Generally, the treatments were less inhibitory to colony development than conidial germination, which was most inhibited for *C. macrodidymum* and least for *C. destructans* (Chapter 5). The effects on isolates were generally similar within a species and overall the mustard meal was the most effective treatment.

In the field experiment (Chapter 5) reductions in disease incidence in grapevine cuttings grown in infested soil was greatest when the soil was treated with mustard meal ($P<0.10$), but also greatly reduced in soils which had chopped mustard plants incorporated into the soil twice after flowering, reductions being 36 and 27%, respectively. Despite the lack of statistical significance at the 5% level, the potential of mustard biofumigation to control black foot disease is indicated. This control method offers a sustainable solution to nursery propagators or vineyard owners who have some spreading patches of vines with this disease. The incorporation of mustard meal requires little preparation time and provides a relatively quick effect. The more economic option is the sowing of mustard seed twice, with successive incorporation into the soil. This method requires a longer period for growth (approximately six weeks each time) and two cultivations but has the advantage of also increasing soil organic matter and would be suitable for fallow nursery land.

6.2 Limitations and future work

The *in vitro* studies clearly showed that the three isolates used for each species varied in their responses to the control treatments and that there was variation between species. For *C. destructans* and *C. macrodidymum* this interspecific variation may have been associated with their inaccurate identification since recent publications used new molecular techniques that reclassified these species into multiple new species. Chaverri *et al*. (2011) divided the *Neonectria* into four groups: 1) *Neonectria*/*Cylindrocarpon* sensu stricto 2) *Rugonectria* 3) *Thelonectria* and 4) *Ilyonectria.* Cabral *et al.* (2012a) introduced 12 new taxa within the *Ilyonectria radicicola* (*C. destructans*) species complex and more recently Cabral *et al*. (2012b) demonstrated that there are six monophyletic species within the *Ilyonectria macrodidyma* complex. However, the purpose of this research was to develop control strategies which were effective against a broad range of *Cylindrocarpon* species, and so the presence of multiple other species within the three originally designated species is of relatively low importance for the overall outcome.

The field experiments showed reductions in infection levels but not complete protection or elimination of infection, which could not be attributed to species or isolates as the inoculum was mixed and there was no easy way to track the isolates once they were placed in the soil. However, since vineyard soils are likely to contain numerous different isolates and species, the recommended control strategies should be effective against many or all isolates. The difference in effect between the Auckland and Blenheim sites for the same chemical and biological pre-planting treatments may have been as a result of species distribution or abiotic and biotic environmental differences. Furthermore, the fungicide experiments conducted at Lincoln University (HRA) were compromised by either lack of soil-borne *Cylindrocarpon* inoculum or frost (Chapter 2). Further experiments testing captan and cyprodinil + fludioxonil (Switch™) in infested soil, with a range of inoculum concentrations, could provide more evidence of the efficacy of these fungicides for control of black foot disease of grapevines.

The HWT provided excellent control of *Cylindrocarpon* spp. (Chapters 3 and 4). However, the results of this study did not agree with all overseas research, possibly because of differences in climate and plant varieties. Since only three grafted rootstock variety combinations were tested, a repeat study could be undertaken which includes 5C grafted to cv. Pinot noir, to

clarify the apparent effects shown in this study. Since other variety combinations are used in New Zealand viticulture, other varieties should also be included in the repeat experiment. Although no rootstocks have been found completely resistant to infection by *Cylindrocarpon* spp., further investigation into rootstock and cultivar interactions could be beneficial as it may establish combinations that have greater resistance to development of black foot disease when planting into at risk soils, or show improved responses to HWT. The originality of the research which investigated HWT effects to root carbohydrate concentrations made it difficult to access comparative data and draw conclusions or gather insights. A longitudinal study using grafted grapevines may provide further insight into effects of HWT and cold storage on carbohydrate concentrations in grape vine roots and the role that carbohydrates play in plant health.

Biofumigation presented a potential control of black foot disease which was supported by the *in vitro* experiment, however these early field experiments need to be repeated on a larger scale, potentially with varieties that release more ITC compounds such as Caliente, to reduce variability and provide statistically robust data.

Management recommendations provided to grape growers in the past have been based on the prevention and/or correction of predisposing stress factors, such as the planting of disease free plant material and use of free-draining soils. The experiments in this research have provided further strategies to reduce levels of the pathogen in the soil, to protect young plants from infection and to eliminate the pathogen from within the plants before they are planted out. This research has therefore provided New Zealand grape growers and nursery propagators with more tools for development into an integrated and sustainable control system.

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Appendix A

Isolates and additional information for Chapter 1

A.1 Isolates used in Auckland (Experiment 1) and Blenheim (Experiment 2).

Lincoln University Plant Pathology number (LUPP)	**Species**	**Geographic region of origin**
959	*Cylindrocarpon liriodendri*	Hawkes Bay
982	*Cylindrocarpon destructans*	Waipara
986	*Cylindrocarpon liriodendri*	Waipara
1047	*Cylindrocarpon macrodidymum*	Marlborough
1132	*Cylindrocarpon destructans*	Marlborough

A.2 Isolates used for all other experiments

Name	LUPP No.	Species	Geographic origin and reference number	Colony morphology on potato dextrose agar (PDA)
1D	1071	*Cylindrocarpon destructans*	Marlborough MAR 7d	Poor sporulation small quantities of macro conidia many micro-conidia. Often only one septate. Dark brown colonies, concentric growth pattern.
2D	1022	*Cylindrocarpon destructans*	Central Otago CO 5a	Good sporulation mostly macro-conidia. Dark brown colonies, concentric growth pattern
3D	989	*Cylindrocarpon destructans*	Waipara WPA 2a	Poor sporulation mixed macro & micro conidia. Often only one septate. Dark brown colonies, concentric growth pattern
1L	1000	*Cylindrocarpon liriodendri*	Central Otago CO 3b	Large macro-conidia good sporulation. Light cream colonies, concentric growth pattern
2L	1102	*Cylindrocarpon liriodendri*	Marlborough MAR 10j	Large macro-conidia good sporulation. Light cream colonies, concentric growth pattern
3L	953	*Cylindrocarpon liriodendri*	Hawkes Bay HB 1a	Large macro-conidia good sporulation. Light cream colonies, concentric growth pattern
1M	974	*Cylindrocarpon macrodidymum*	Hawkes Bay HB 6e	Medium sized spores good sporulation, slow germination. Dark brown colonies, irregular radial growth, with white/yellow fluffy surface
2M	1039	*Cylindrocarpon macrodidymum*	Gisborne GIS 3d	Medium sized spores good sporulation, mostly macro-conidia. Dark brown colonies, irregular radial growth, with white/yellow fluffy surface
3M	1120	*Cylindrocarpon macrodidymum*	Marlborough MAR 14c	Medium sized spores good sporulation, slow germination. Dark brown colonies, irregular radial growth, with white/yellow fluffy surface

A.3 Impact of mycorrhizal colonisation on grapevine establishment in *Cylindrocarpon* infested spoil.

IMPACT OF MYCORRHIZAL COLONISATION ON GRAPEVINE ESTABLISHMENT IN *CYLINDROCARPON* INFESTED SOIL

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ABSTRACT

The effects of arbuscular mycorrhizae (AM), *Glomus mosseae* and *Acaulospora laevis*, on growth of six grape rootstock varieties and their infection by *Cylindrocarpon* species were investigated in a greenhouse experiment that challenged the mycorrhizal plants with a mixed inoculum of *Cylindrocarpon* spp. Both species of AM had beneficial effects ($P<0.05$) on rootstock growth but this effect was specific for particular rootstock and AM combinations. Generally, *G. mosseae* had a more consistent positive effect and *A. laevis* was the only species to decrease plant growth in some rootstock varieties. Subsequently, two field trials conducted in Auckland and Blenheim used a mixture of the two AM species and two rootstock varieties. After 9 months, compared to control plants mycorrhizal plants had similar incidence of *Cylindrocarpon* infection at both sites ($P>0.05$), but greater root dry weights in Auckland (214%; $P<0.05$) and Blenheim (only 22%; $P>0.05$). *G. mosseae* clearly has potential for improving health and growth of young grapevine plants.
Keywords: mycorrhizal, *Glomus mosseae*, *Acaulospora laevis*, colonisation, *Cylindrocarpon*, black foot disease, grapevines, rootstocks.

INTRODUCTION

Cylindrocarpon black foot (BF) disease of grapevines (*Vitis* spp.) has been identified as a major cause of death of young vines in South Africa, North and South America, Australia, New Zealand and several Mediterranean countries in the last 5–10 years. Three species of *Cylindrocarpon* Wollenw., *C. destructans* (Zinnsm.) Scholten, *C. macrodidymum* Schroers, Halleen & Crous (Halleen et al. 2004) and *C. liriodendri* JD MacDon. & EE Butler, (MacDonald & Butler 1981) were reported to be associated with the disease in New Zealand vineyards (Bleach et al. 2007). Symptoms include weak shoot growth, abnormal root development, necrotic root crowns, dark discolouration of vascular tissue and internal necrosis extending from the bark to the pith of affected rootstocks. The primary source of inoculum is the soil and infection is usually via the roots and basal end of the trunk. Research has shown that all rootstock varieties commonly used in New Zealand vineyards are susceptible to *Cylindrocarpon* spp. (Jaspers et al. 2007).

Arbuscular mycorrhizae (AM) are believed to suppress infection by root pathogens mainly through competition with the pathogens for infection points on the roots (Muchovej 2004). Results from studies carried out in California (Gubler & Petit 2006) suggested that preplant applications of AM may reduce BF disease in the nursery and in the vineyard. This greenhouse experiment at Lincoln University investigated colonisation of six common rootstocks by two mycorrhizal species, *G. mosseae* and *A. laevis*, and their potential to protect against BF disease. Field experiments in both Auckland and Marlborough tested the effectiveness of mycorrhizae compared with four other soil treatments to enhance establishment of young rootstock plants.

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MATERIALS AND METHODS

Greenhouse experiment

Callused cuttings of six rootstock varieties, 101-14, 3309, 420A, 5C, Riparia Gloire and Schwarzmann, were planted into a soil-free potting mix deficient in phosphorus in 2.5 litre plastic containers and laid out in a randomised design, with six replicates for each treatment combination. The six rootstocks were inoculated with *G. mosseae* or *A. laevis*, 150 spores per vine, as previously described (Brundrett et al. 1996). The plants were grown for 7 months and the root samples were assessed for mycorrizal colonisation by staining with trypan blue followed by microscopic examination, as previously described (Brundrett et al. 1996). The plants were then inoculated with 60 ml of a mixed conidial suspension (10^6/ml) of *C. destructans*, *C. macrodidymum* and *C. liriodendri*. The plants were grown in a greenhouse and harvested 5 months after inoculation with *Cylindrocarpon* spp. Initially, the experimental design included pathogen free control plants for each of the three treatments (no mycorrhizae, *G. mosseae* only, *A. laevis* only). However, it soon became apparent that splash dispersal during watering had resulted in all treatments being infected with *Cylindrocarpon* spp. As the amount of inoculum was not consistent, the pathogen free controls were removed from the analysis.

Field experiment

Rootstock cuttings of 101-14 and 5C were planted into trays of pumice and placed on heat pads at 25°C for 5 weeks to develop calli. For the AM treatment, the cuttings were inoculated by adding AM spores (200 spores of each species per litre) to the pumice. A sample of roots was taken from these plants to verify AM colonisation.

The field sites in Blenheim and Auckland were previously used as grapevine nurseries and known to be infested with *Cylindrocarpon* spp. They had also been inoculated for an experiment the previous year with 20 ml of *C. destructans*, *C. macrodidymum* and *C. liriodendri* (mixed conidia, 10^6/ml) injected at 24 positions/m^2 to a depth of about 15 cm giving a conidia concentration of 4.8×10^8 conidia/m^2. Prior to planting the soils were cultivated, thereby mixing the remaining inoculum (if any) through the site. In Blenheim the same site was used as before but in Auckland movement of a fence led to misdirection, and the experimental site was later found to be adjacent to and about 3 m from the initially inoculated soil. Therefore, inoculum levels were expected to be lower than in Blenheim but no soil plating was done to determine inoculum levels.

Planting was done in late October 2006 in Auckland and mid November in Blenheim. One month prior to planting, four soil treatments were applied. These were two soil fumigants, (1) Telone C35 (soil fumigant), (2) Chloropicrin (soil fumigant), both applied by contractors, and two soil amendments, (3) active compost at 40 kg/m^2 and (4) WormTech™ liquid at 5 litres/m^2. Other treatments were (5) untreated soil and (6) untreated soil with AM treated rootlings. The treatments were replicated six times. The two rootstock varieties were planted as subplots. Within this layout, each sub-plot had a double row of six plants of which the centre eight were assessed and the end plants treated as buffers (6x6x2x8 = 576 plants assessed). The plants were set out in a randomised split plot design, planted according to standard nursery practices, grown for 8 months and then harvested.

Harvest

For both greenhouse and field experiments, grapevine root and shoot dry weight (DW) was recorded. The plant trunks were surface-sterilised, dissected and segments of the basal trunk tissue transferred to potato dextrose agar (Oxoid) as described by Halleen et al. (2003). After 7-10 days incubation at 20°C, *Cylindrocarpon* spp. growing from the plant tissue were identified morphologically and results recorded.

Statistical analysis

In the greenhouse experiment, incidence of infection by *Cylindrocarpon* spp. data for the three treatments (*A laevis* + *Cylindrocarpon*, *G. mosseae* + *Cylindrocarpon* and *Cylindrocarpon* only) were back transformed prior to analysis using the generalized linear mixed model (GLMM) to compare disease incidence. All other data were analysed by analysis of variance (ANOVA) using Genstat 9. Comparison of means was conducted with Fishers protected least significant difference (LSD) test at $P<0.05$.

RESULTS AND DISCUSSION

Greenhouse experiment

Table 1 shows that the six rootstocks had very different responses to inoculation with AM and that these responses were variable depending on the specific AM and rootstock combination ($P<0.05$). Shoot DW was significantly improved by inoculating 101-14 with AM, although only *A. laevis* also improved root DW for this rootstock variety. For rootstock 3309, only *G. mosseae* improved shoot DW, whereas both AM increased root DW. In contrast, for rootstock 420A only *A. laevis* increased both root and shoot DW. For rootstocks 5C, Riparia Gloire and Schwarzmann, inoculation with *G. mosseae* significantly increased shoot DW, although the increased shoot mass induced by this AM was only associated with increased root DW in 5C and Riparia Gloire. However, the effect of *A. laevis* was quite different, with significantly decreased shoot DW of 5C, Riparia Gloire and Schwarzmann, and decreased root DW of Riparia Gloire and Schwarzmann. The percentage infection by *Cylindrocarpon* spp. was not significantly different between treatments ($P=0.883$) or between rootstocks ($P=0.057$), even though the values for rootstocks 3309 (0.1%) and Riparia Gloire (0.3%) were very low. The low values for Riparia Gloire and 3309 were primarily due to a complete absence of infection in the *G. mosseae* treatment.

TABLE 1: Shoot and root dry weights (g DW/plant) for grapevine rootstocks inoculated with *Cylindrocarpon* spp. with or without arbuscular mycorrhizae (*Glomus mosseae* or *Acaulospora laevis*) and mean incidence of *Cylindrocarpon* infection (%) of rootstocks across all treatments.

		Rootstock variety					
		101-14	3309	420A	5C	R.Gloire	Schwarz
Shoot DW							
	Control	18.62a[1]	9.80a	13.52a	16.97a	12.02a	18.52a
	A. laevis	21.69b	10.18a	15.46b	15.09b	9.57b	11.47b
	G. mosseae	22.73b	11.76b	13.25a	21.94c	14.60c	21.15c
	LSD=1.68						
Root DW							
	Control	18.59a	12.90a	12.62a	15.73a	15.42a	15.90a
	A. laevis	22.32b	15.73b	15.58b	22.22b	10.76b	11.40b
	G. mosseae	17.79a	15.87b	11.78a	20.42b	23.89c	16.60a
	LSD=2.02						
Cylindrocarpon infection							
	P=0.057	16.8	0.3	14.0	17.8	0.1	8.8

[1]Means for the shoot or root DW sharing the same letters are not significantly different at $P<0.05$.

Grapevine rootstocks are well known for their different biological traits and variable colonisation by AM (Schreiner 2003). Studies on the ability of AM to reduce the deleterious effects of soil-borne pathogens have also shown that these effects vary with the plant host and AM combination (Azcón-Aguilar & Barea 1996). In this study, grapevine rootstocks inoculated with AM showed some variation in their resistance to infection by *Cylindrocarpon* spp. Generally, *G. mosseae* had a greater beneficial effect than *A. laevis* across all rootstocks, and only *A. laevis* had a negative effect on the growth

of some rootstocks. In similar work, Petit & Gubler (2006) showed that inoculation with *G. intraradices* decreased disease severity in grapevines infected by *Cylindrocarpon* spp. The mechanisms by which AM protect plants against soil-borne pathogens is poorly understood, but is thought to include increasing nutrient uptake, competition for infection sites and changes to root ultrastructure (Azcón-Aguilar & Barea 1996). In this study the AM associations improved plant growth, although the exact mechanism by which this occurred is unclear.

Field experiment

The soil treatments for the Auckland and Blenheim experiments did not significantly reduce *Cylindrocarpon* infection incidence ($P>0.05$) of the rootstock plants nor were any differences seen between varieties ($P>0.05$). In Blenheim approximately 30% of the plants died due to hot, dry conditions after planting and were replaced within 4-5 weeks of the initial planting. Early plant survival data were not recorded for the Auckland site. At harvest, only plants with both roots and shoots were classified as live plants. Survival of plants for the mycorrhizal (57%), Telone C35 (45%) and control (49%) treatments were significantly different, and these levels were consistent between the two rootstocks. AM are known to alleviate drought stress and this effect is thought to be variable between AM species and isolates (Augé 2001). In the present research, inoculation of rootstocks with *A. laevis* and *G. mosseae* seemed to have increased survival during the drought conditions experienced in Blenheim, as reported by Salinger (2006, 2007), who described significant soil moisture deficits (more than 110 mm and 130 mm) that had persisted in central Marlborough in 2006 and 2007 respectively. However, it is unclear whether the effect in the present research was attributable to one of the AM species or the combination. Future experiments could address the relative effect of each AM species on increasing drought tolerance in grapevines.

All of the 576 plants harvested from each site were assessed, of which 85% were alive in Auckland and 73% in Blenheim (Figures 1 & 2). The disease incidence for Auckland plants was low (<25%) compared to Blenheim plants (>50%), probably because of the lower soil inoculum, since they were planted alongside and not in the site that had been inoculated with *Cylindrocarpon* spp. the previous year. The treatments at both these sites did not have a significant effect on *Cylindrocarpon* incidence, although there were large differences between some treatments. For example, there was a trend ($P=0.13$) for the fumigants chloropicrin and Telone C35 to reduce *Cylindrocarpon* incidence by 16 and 17% respectively, in Blenheim and by 24 and 42%, respectively, in Auckland ($P=0.096$), compared with the untreated control. Of the biological treatments, only the Wormtech™ product caused a trend for reducing *Cylindrocarpon* incidence (by 30%; $P=0.13$) and only in the Blenheim site.

The dry weight assessments indicated significant ($P<0.05$) effects of the treatments on plant growth at the Auckland site (Fig. 1), with AM causing far greater increases in root DW (214%), relative to the control, than that recorded for the WormTech™ (26%) and active compost (42%) treatments. In Blenheim, there were no significant treatment effects ($P>0.05$), although there were some trends with mycorrhizal colonisation and Telone C35 treatments, both causing 9% increased root DW (Fig. 2). The overall trends in shoot weights tended to reflect those of the root weights. Clearly precolonisation of rooted cuttings prior to planting led to an increased root mass, which is likely to contribute to better establishment of these plants in the field. The rootstocks used in the field trial were 101-14 and 5C, which had responded well to both *A. laevis* and *G. mosseae* in the greenhouse trials. The field soil will also have contained AM spores and future research could determine whether colonisation by field AM was stimulated or out-competed by the inoculated strains.

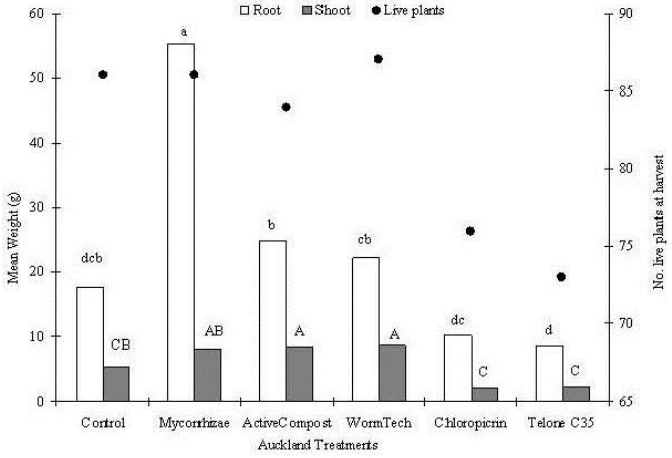

FIGURE 1: Mean root and shoot dry weights and the number of live plants in the Auckland field experiment. Means with the same letter are not significantly different (P<0.001).

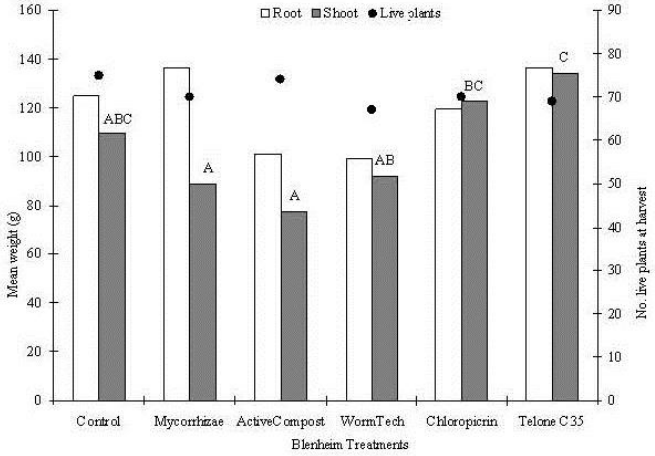

FIGURE 2: Mean root and shoot dry weights and the number of live plants in the Blenheim field experiment. Shoot means with the same letter are not significantly different (P<0.05). Root means were not significantly different (P>0.05).

CONCLUSION

The greenhouse experiment showed that plants colonised by AM had improved vigour, with the extent of the effect being specific for particular rootstock and AM combinations. These data indicate that the relationship between the plant and symbiont is complex and can be influenced by the traits of individual cultivars. Future research could investigate changes in the mycorrhizosphere to elucidate the mechanism by which *Cylindrocarpon* spp. are inhibited and whether these and other AM species can protect against other soil-borne diseases. In addition, it would be useful to know whether the different AM species can exhibit synergy or whether they compete with each other for the ecological niche, with one or some species preferentially colonising the roots.

The field experiments indicated that plants colonised by AM had dramatically increased root mass over other treatments. Increased root mass is likely to contribute to better establishment and healthier plants that have a greater ability to withstand environmental stressors such as drought. The experiments demonstrated that, not only are AM a realistic option for control of BF disease, they can also contribute to greater health of young plants under field conditions.

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A.4 Survey for black foot disease in New Zealand vineyards

SURVEY FOR BLACK FOOT DISEASE IN NEW ZEALAND VINEYARDS

Bleach, C. M., Jones, E. E., and Jaspers, M. V. (2007). *Survey for black foot disease in New Zealand vineyards.* Paper presented at the 13th Annual Conference, Romeo Bragato, Ellerslie Event Centre, Auckland, New Zealand.

INTRODUCTION

In recent years, vineyards throughout the world have reported increasing numbers of declining young grapevines, especially in newly established and replanted sites. The decline symptoms usually include stunting, chlorosis, late bud break and sometimes death of vines. Two diseases, 'Petri vine decline' and 'black foot', have been recognized as the major causes of the decline, with black foot disease (BF) being the more serious of the two as it causes vine death. The *Cylindrocarpon* spp. that cause BF disease are common soil inhabitants, occurring as saprophytes or weak pathogens, often associated with roots of herbaceous woody plants (1). The epidemiology of this disease is not well understood and overseas research has concentrated on identifying the causal agents and effective management strategies. In New Zealand, the severity of BF and the pathogen(s) responsible had not been investigated and so a research project was initiated in February 2005 at Lincoln University to determine the prevalence of BF, the *Cylindrocarpon* spp. responsible and the cultural factors associated with disease incidence.

MATERIALS AND METHODS

Grape growers from New Zealand were invited to send symptomatic BF grapevines to Lincoln University for assessment, and asked to provide information on the grapevine scion and rootstock, age and growing conditions. The 141 vines supplied by 49 growers were surface sterilized, dissected and the symptomatic root and trunk tissue transferred to potato dextrose agar as previously described (2). After 7-10 days incubation at 20ºC, the *Cylindrocarpon* isolates were identified morphologically (3). Single spore colonies were grown on synthetic nutrient-poor agar (1) and sent to Stellenbosch University, South Africa, for molecular identification (3).

RESULTS AND DISCUSSION

Of the 141 grapevines analysed, 204 *Cylindrocarpon*-like fungi were detected in 121 vines. The decline symptoms included delayed bud burst, stunted growth, yellowing of leaves and early senescence (Figure 1).

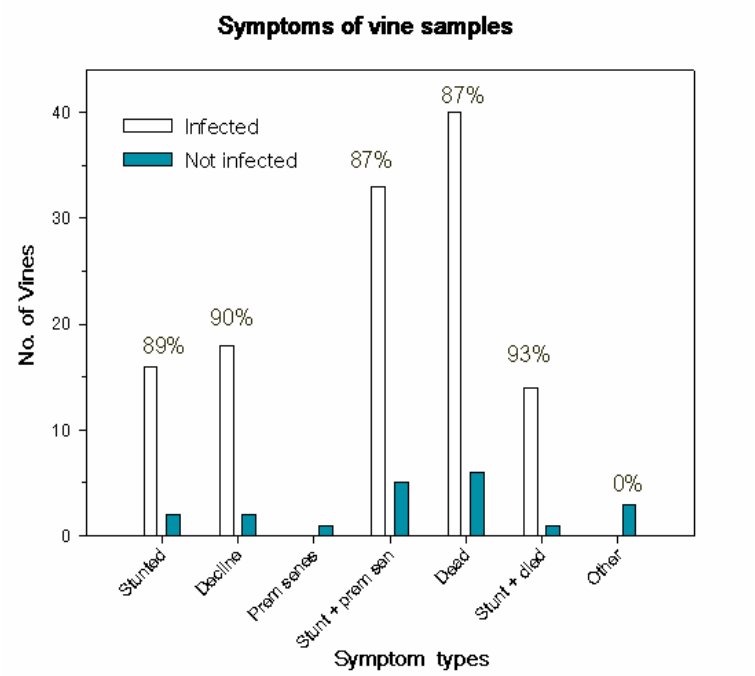

Figure 1. Vine decline symptoms displayed by the *Cylindrocarpon* infected and healthy vine samples sent by growers, with percent infection shown above the infected bars for each symptom type.

In Marlborough (Figure 2) disease incidence was greatest in young vines 1-4 years old (67%), but occurred at low levels in vines of all ages (up to 12 years). The disease was more prevalent in Marlborough, but this was due to the increased plantings in that region during the last 2-3 years. Age of vines at first harvest did not affect BF incidence, with samples from northern regions usually having two seasons growth prior to harvest and those from southern areas three seasons growth (data not shown). These results seem to demonstrate that the two and three year plants were both at a similar (minimum) stage of development when they were considered by growers to be ready for a first crop.

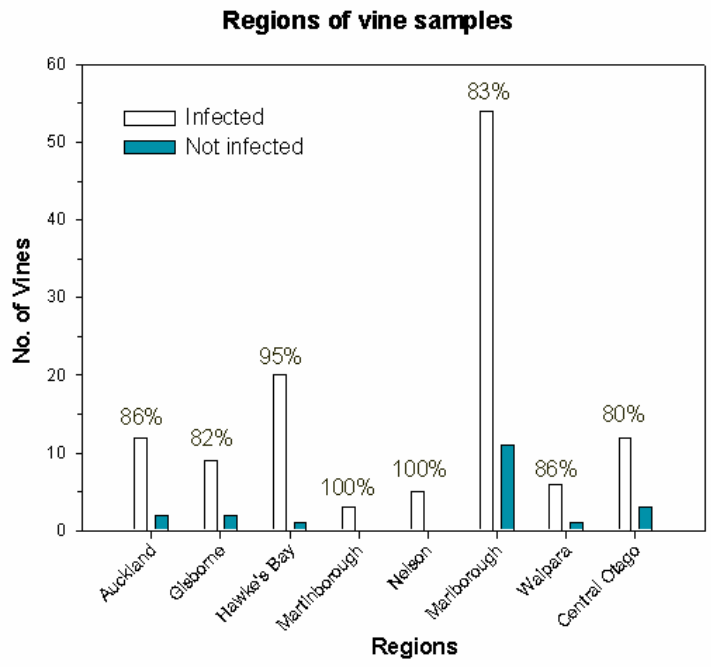

Figure 2. Regional distribution of *Cylindrocarpon* infected and uninfected vine samples supplied showing percentage of actual vines infected.

BF infection was found in all soil types and was isolated from all the eight rootstock varieties represented in the survey. Information supplied by growers indicated that the most common soil type (Figure 3) was sandy/stony silt loam however all soil types had similar infection levels.

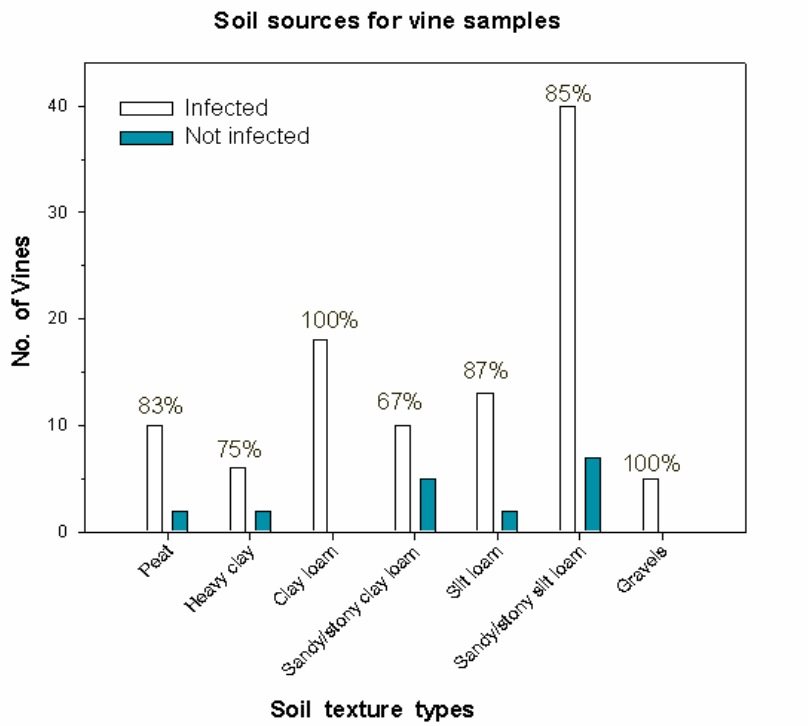

Figure 3. Soil types from all regions showing percentage of actual vines infected.

The most common rootstock sent by growers (Figure 4) was 101-14, however all samples supplied had similar infection levels (75-90%) except rootstock varieties 3309 (60%) and 125AA (50%).

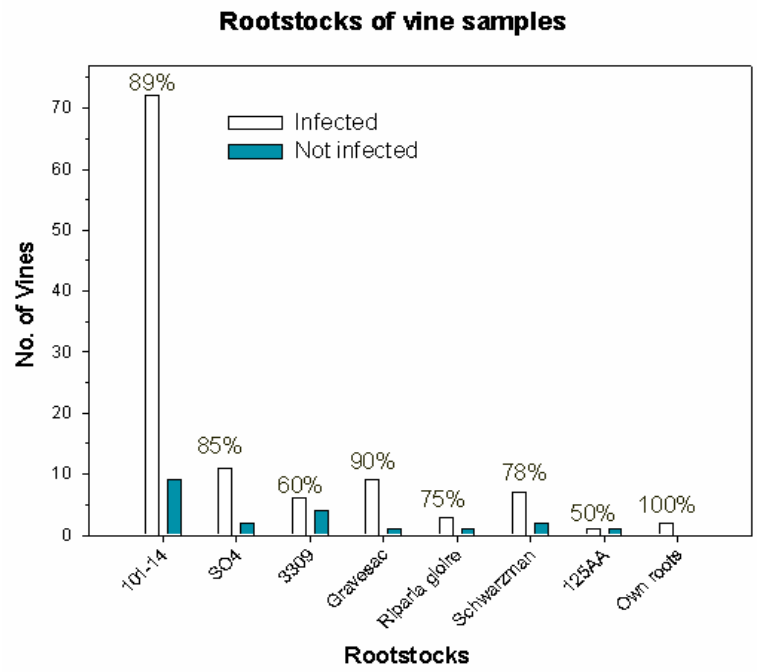

Figure 4. Rootstock samples supplied showing percentage of actual vines infected.

CONCLUSIONS

The results indicate that 'Cylindrocarpon black foot' is present in all New Zealand vineyard regions sampled, with similar disease incidences across all rootstocks and regions. Young vines had greater incidence of disease, which is consistent with overseas reports, but despite the wide range of vineyard soil types sampled, there was no correlation found with heavy soil. This is in contrast to some overseas reports of the disease being more prevalent in heavy, low-lying soils. Initial identification of the *Cylindrocarpon* spp. by fungal morphology indicated that several species were responsible for grapevine casualties in New Zealand nurseries and vineyards. These have been identified by South African researchers, using the T1 and T2 primers, which targeted the betatubulin region of the genome, as *C. destructans, C. liriodendri* and *C. macrodidymum*. Since the disease is so widespread, an investigation is currently under way to find suitable control measures to prevent infection of plants or to eradicate inoculum from soil.

ACKNOWLEDGEMENTS

New Zealand Winegrowers for funding this project and the grape growers who participated in the survey.

REFERENCES

1. Brayford D (1993). *Cylindrocarpon*. In 'Methods for research on soil borne phytopathogenic fungi.'(Eds LL Singleton, JD Mihail, M Rush) pp. 103-106. (APS Press: St Paul, USA).

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3. Halleen F, Schroers H-J, Groenewald JZ, and Crous PW (2004). Novel species of *Cylindrocarpon* (*Neonectria*) and *Campylocarpon* gen. nov. associated with black foot disease of grapevines (*Vitis* spp.). *Studies in Mycology, 50*, 431-455.

A.5 Survey for black foot decline in New Zealand vineyards

Bleach, C. M., Jones, E. E., and Jaspers, M. V. (2006). *Survey for black foot decline in New Zealand vineyards.* Paper presented at the 4th Australasian Soilborne Diseases Symposium, Queenstown, New Zealand.

INTRODUCTION

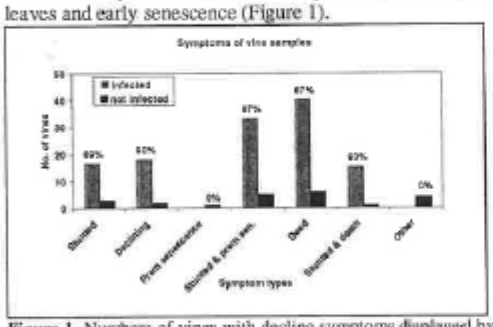
In recent years, vineyards throughout the world have reported increasing numbers of declining young grapevines, especially in newly established and replanted sites. The decline symptoms usually include stunting, chlorosis, late bud break and sometimes death of vines. Two diseases, 'Petri vine decline' and 'black foot', have been recognized as the major causes of the decline, with black foot (BF) being the more serious of the two as it causes vine death. The *Cylindrocarpon* spp. that cause BF are common soil inhabitants, occurring as saprophytes or weak pathogens, often associated with roots of herbaceous woody plants (1). The epidemiology of this disease is not well understood and overseas research has concentrated on identifying the causal agents and effective management strategies. In New Zealand, the severity of BF and the pathogen(s) responsible had not been investigated and so a research project was initiated in February 2005 at Lincoln University to determine the prevalence of BF, the *Cylindrocarpon* spp. responsible and the cultural factors associated with disease incidence.

MATERIALS AND METHODS

Grapegrowers from throughout New Zealand were invited to send symptomatic BF grapevines to Lincoln University for assessment, and asked to provide information on the grapevine scion and rootstock, age and growing conditions. Tissue from the 141 vines supplied by 49 growers was surface sterilized, dissected and the symptomatic root and trunk tissue transferred to potato dextrose agar as previously described (2). After 7-10 days incubation at 20°C, the *Cylindrocarpon* isolates were identified morphologically (3). Single spore colonies were grown on synthetic nutrient-poor agar (1) and sent to Stellenbosch University, South Africa, for molecular identification (3).

RESULTS AND DISCUSSION

Of the 141 grapevines analysed, 204 *Cylindrocarpon*-like fungi were detected in 121, and these isolates were sent to South Africa for molecular identification. BF infection was found in all soil types and was isolated from all the eight rootstock varieties tested. Decline symptoms included delayed bud burst, stunted growth, yellowing of leaves and early senescence (Figure 1).



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The disease was more prevalent in Marlborough (Figure 2), but this was due to the increased plantings in that region during the last 2-3 years. Disease incidence was also greatest in young, 1-4 year old vines (67%), but occurred at low levels in vines of all ages (up to 12 years). Age of vines at first harvest did not affect BF incidence, with samples from northern regions usually having two seasons growth prior to harvest and those from southern areas three seasons growth (data not shown). These results seem to demonstrate that the 2 and 3 year plants were both at a similar (minimum) stage of development when they were considered by growers to be ready for a first crop.

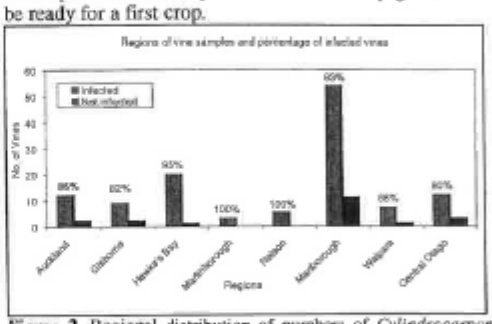

Figure 2. Regional distribution of numbers of *Cylindrocarpon* infected and uninfected vine samples supplied showing percentage of actual vines infected.

CONCLUSIONS

The results indicate that 'Cylindrocarpon black foot' is present in all New Zealand vineyard regions sampled, with similar disease incidences across all rootstocks and regions. Young vines had greater incidence of disease, which is consistent with overseas reports, but despite the wide range of vineyard soil types sampled, there was no correlation found with heavy soil, in contrast to some overseas reports. Initial identification of the *Cylindrocarpon* spp. by fungal morphology indicated that several species were responsible for grapevine casualties in New Zealand nurseries and vineyards, and confirmation of their identification is currently under way. Since the disease is so widespread, an investigation to establish suitable control measures to prevent or eradicate infection is warranted.

ACKNOWLEDGEMENTS

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REFERENCES

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Appendix B

Media and potting mix recipes

B.1 Agar

B.1.1 Potato dextrose agar (PDA)

39 g of PDA (Oxoid Ltd, Basingstoke, UK) was added to 1 L of reverse osmosis water (ROW) and autoclaved for 20 min at 121°C and 15 psi.

Potato dextrose agar (PDA) amended with chloramphenicol

39 g of PDA (Oxoid Ltd, Basingstoke, UK) was added to 1 L of reverse osmosis water (ROW). Under a fume hood, 0.25 g of chloramphenicol was added to the solution and autoclaved for 20 min at 121°C and 15 psi.

B.1.2 Spezieller Nährstoffarmer Agar (SNA)

1.0 g KH_2PO_4

1.0 g KNO_3

500 mg $MgSO_4.7H_2O$

500 mg KCl

200 mg Glucose

200 mg Sucrose

20 g Agar

1 L Distilled water

All ingredients except agar were dissolved in 1 L of SDW and the pH adjusted to 6-6.5 with dilute HCl. The agar was added and dissolved before the solution was autoclaved (20 min at 121°C and 15 psi) and allowed to cool to 50°C (Nirenberg, 1976 in Brayford, 1993). The SNA was poured into 90 mm Petri dishes and once set two pieces (~1 cm^2) of sterile filter paper were placed onto the agar surface to enhance sporulation.

B.1.3 Malt extract agar (MEA)

Malt extract (Maltexo)	20 g
Agar	20 g
Water	1 L

The malt extract (Maltexo, Lion New Zealand) was dissolved in 1 L of ROW. The agar was added and dissolved before the solution was autoclaved (20 min at 121°C and 15 psi) (Johnston and Booth, 1983). If required, 0.25 g of chloramphenicol was added to the solution before and autoclaving.

B.1.4 Potting mix

80% horticultural bark (grade 2): 20% pumice (Southern Horticultural Products Ltd South, Christchurch) amended with 2 g/L Osmocote Exact® plus Hydraflo® 1 g/L (Everris, New Zealand) and agricultural lime 1 g/L (Ravensdown New Zealand Ltd) per 2.5 L container.

Appendix C

Statistical analysis and additional data for Chapter 2

C.1 Fungicides used in the *in vitro* and *in vivo* experiments and their mode of action and pathogen effect (Young, 2010).

Trade Name and *group*	Active Ingredient	Registered for grapes	Mode of action and pathogen effects
Amistar *Strobilurin*	Azoxystrobin	Yes	Systemic translaminar and protectant action having additional curative and eradicant properties. Respiration inhibitor and anti-sporulant properties.
Acanto *Strobilurin*	Picoxystrobin	No	Systemic. Inhibits protein synthesis, interferes with respiration in plant pathogenic fungi inhibiting spore germination and mycelium growth.
Captan *Phthalimide*	Captan	Yes	Non-systemic with protective and surface curative action against invading fungi.
Sporekill *Biocide*	Didecyldimethyl-ammonium chlorine	Yes	Non-systemic. Broad spectrum, biocide disinfectant kills spores by contact action.
Switch *Anilino-Pyrimidine + Phenyl- Pyrrole*	Cyprodinil + Fludioxonil	Yes No	Cyprodinil - Systemic, absorbed through foliage. Inhibits protein synthesis. Fludioxonil - Non-systemic with long residual activity. Inhibits transport-associated phosphorylation of glucose, reducing mycelial growth. Multi -site action inhibiting spore germination penetration of the germ tube.
Maxim *Phenyl- Pyrrole*	Fludioxonil	No	Non-systemic with long residual activity. Inhibits transport-associated phosphorylation of glucose, reducing mycelial growth.
MBC-500 *Benzimidazole*	Carbendazim	Yes	Systemic with curative and protectant activity. Inhibition of mitosis and cell division. Transported via the sap stream in an upward and outward direction.
Score *Triazole Demethylation inhibitor (DMI)*	Difenoconazole	No	Systemic with preventative and curative action. Disrupts membrane function. Penetrates into green tissue and acts on developing fungal infection during the incubation period.
Nustar *Triazole (DMI)*	Flusilazole	No	Broad-spectrum, systemic with protective and curative action. Translocation through rising sap. Stops germ tube growth and Sporulation.
Mirage 450 *Imidazole (DMI)*	Prochloraz	No	Broad-spectrum with protectant and eradicant properties. Disrupts membrane function.
Chlorothalonil *Chloro-nitrile*	Chlorothalonil	Yes	Non-systemic, broad-spectrum, foliar action with some protectant properties. Acts by preventing spore germination and zoospore motility.
Folicur WG *Triazole (DMI)*	Tebuconazole	No	Systemic with protective, curative and eradicant action. Disrupts membrane function. Absorbed by leaves and stems and translocated through plant to protect new growth.

C.2 Statistical analysis and additional data for Chapter 2

C.3 *In vitro* experiments

C.3.1 Analysis by general linear model of the effect of fungicides on in vitro mycelium growth of nine isolates of three Cylindrocarpon species.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Species	60523.99	2	30262.00	579.53	<0.001
Chemical	173988.84	11	15817.17	302.90	<0.001
Species x fungicide	272199.54	22	12372.71	236.94	<0.001
Error	3759.72	72	52.22		

C.3.2 Analysis by general linear model of the effect of fungicides on *in vitro* conidium germination of nine isolates of three *Cylindrocarpon* species.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Species	3464.0	2	1732	1.68	0.193
Chemical	125455.0	11	11405	11.09	<0.001
Species x fungicide	21548.0	22	979	0.95	0.531
Error	74015	72	1028		

C.4 *In vivo* experiments Auckland statistical analysis (2006)

C.4.1 Analysis by general linear model of the effect of treatments on disease severity.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	1050.83	6	175.14	5.40	0.001
Variety	84.00	1	84.00	4.31	0.045
Treatment x variety	58.83	6	9.80	0.50	0.801
Error	681.16	35	19.46		

C.4.2 Analysis by general linear model of the effect of treatments on disease incidence at 0 cm.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	152.14	6	25.36	6.06	0.000
Variety	26.30	1	26.30	11.97	0.001
Treatment x variety	12.28	6	2.05	0.93	0.485
Error	76.92	35	2.20		

C.4.3 Analysis by general linear model of the effect of treatments on disease incidence at 5 cm.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	35.95	6	5.99	2.57	0.040
Variety	0.30	1	0.30	0.22	0.639
Treatment x variety	5.62	6	0.94	0.70	0.649
Error	46.58	35	1.33		

C.4.4 Analysis by general linear model of the effect of treatments on dead plants.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	83.81	6	13.97	8.93	0.000
Variety	1.71	1	1.71	1.70	0.201
Treatment x variety	12.95	6	2.16	2.14	0.073
Error	35.33	35	1.01		

C.4.5 Analysis by general linear model of the effect of treatments on dead plants with *Cylindrocarpon* spp. infection.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	2.571	6	0.43	1.765	0.140
Variety	1.44	1	1.44	4.76	0.036
Treatment x variety	1.48	6	0.25	0.814	0.567
Error	10.583	35	0.30		

C.4.6 Analysis by general linear model of the effect of treatments on root dry weight (g).

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	4785.42	6	797.57	4.15	0.004
Variety	1521.07	1	1521.07	8.72	0.006
Treatment x variety	371.36	6	61.90	0.355	0.902
Error	6104.02	35	174.40		

C.4.7 Analysis by general linear model of the effect of treatments on shoot dry weight (g).

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	678.50	6	113.08	4.53	0.002
Variety	469.88	1	469.88	15.67	0.000
Treatment x variety	277.55	6	46.26	1.543	0.193
Error	1049.40	35	29.98		

C.5 *In vivo* experiments Blenheim statistical analysis (2006)

C.5.1 Analysis by general linear model of the effect of treatments on disease severity.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	814.98	6	135.83	6.35	0.000
Variety	0.107	1	0.107	0.005	0.946
Treatment x variety	137.98	6	22.99	1.012	0.423
Error	795.42	35	22.73		

C.5.2 Analysis by general linear model of the effect of treatments on disease.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	145.98	6	24.33	12.74	0.000
Variety	12.19	1	12.19	5.19	0.029
Treatments x variety	12.64	6	2.10	0.898	0.508
Error	82.17	35	2.348		

C.5.3 Analysis by general linear model of the effect of treatments on disease incidence at 5 cm.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	44.476	6	7.413	6.78	0.000
Variety	0.964	1	0.964	0.576	0.453
Treatment x variety	20.952	6	3.492	2.086	0.080
Error	58.58	35	1.67		

C.5.4 Analysis by general linear model of the effect of treatments on disease incidence at 5 cm.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	38.238	6	6.373	2.090	0.084
Variety	394.33	1	394.33	235.26	0.000
Treatment x variety	25.00	6	4.167	2.486	0.041
Error	58.67	35	1.68		

C.5.5 Analysis by general linear model of the effect of treatments on dead plants.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	38.24	6	6.37	2.09	0.084
Variety	394.33	1	394.33	235.26	0.000
Treatment x variety	25.00	6	4.17	2.486	0.041
Error	58.667	3	1.68		

C.5.6 Analysis by general linear model of the effect of treatments on dead plants with *Cylindrocarpon* spp. infection.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	22.95	6	3.82	2.72	0.031
Variety	19.05	1	19.05	18.52	0.000
Treatment x variety	3.95	6	0.66	0.640	0.697
Error	36.00	35	1.03		

C.5.7 Analysis by general linear model of the effect of treatments on root dry weight (g).

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	4577.40	6	762.90	3.73	0.007
Variety	27126.79	1	27126.79	243.08	0.000
Treatment x variety	2108.84	6	351.47	3.149	0.014
Error	3905.90	35	111.60		

C.5.8 Analysis by general linear model of the effect of treatments on shoot dry weight (g).

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	411.03	6	68.50	0.269	0.947
Variety	33116.95	1	33116.95	162.10	0.000
Treatment x variety	1006.08	6	167.68	0.821	0.561
Error	7150.49	35	204.30		

C.6 *In vivo* experiment Lincoln statistical analysis (2006)

C.6.1 Analysis by general linear model of the effect of treatments on root dry weight (g).

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	70.25	5	14.05	5.073	0.004
Error	55.39	20	2.77		

C.6.2 Analysis by general linear model of the effect of treatments on shoot dry weight (g).

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	52.69	5	10.54	4.245	0.009
Error	49.65	20	2.48		

C.7 *In vivo* experiment Lincoln statistical analysis (2007)

C.7.1 Analysis y general linear model of the effect of treatments on disease severity.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	0.00	4	0.00	0.133	0.968
Variety	0.845	1	0.845	3.455	0.088
Treatment x variety	0.761	4	0.190	0.778	0.560
Error	2.93	12	0.24		

C.7.2 Analysis by general linear model of the effect of treatments on disease incidence.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	0.00	4	0.00	0.102	0.980
Variety	0.22	1	0.22	6.783	0.023
Treatment x variety	0.00	4	0.00	0.572	0.688
Error	0.390	12	0.00		

C.7.3 Analysis by general linear model of the effect of treatments on root dry weight (g).

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	62.30	4	15.57	0.567	0.690
Variety	119.50	1	119.50	6.022	0.023
Treatment x variety	24.62	4	6.15	0.310	0.868
Error	396.87	20	19.84		

Appendix D

Statistical analysis for Chapter 3

D.1 *In vitro* Hot Water Treatment experiment

D.1.1 Analysis of variance of the effect of HWT using different temperatures and times combinations on mycelium growth of nine *Cylindrocarpon* isolates.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Isolate	6552.82	8	819.03	3.00	<0.003
HWT temp	458311.44	4	114577.86	420.39	<0.000
HWT time	155670.29	2	77835.15	285.58	<0.001
Isolate x HWT temp	25337.40	32	791.79	2.90	<0.001
Isolate x HWT time	7992.30	16	499.52	1.83	<0.027
HWT temp x HWT time	133570.77	8	16696.35	61.26	<0.001
Isolate x HWT temp x HWT time	38417.35	64	600.27	2.20	<0.001
Error	73588.00	270	272.55		
Species x HWT temp x HWT time	6507.97	16	406.75	1.091	0.362
Error	134200.38	360	372.78		

Species	Mean	Standard error	95% Confidence interval	
			Lower Bound	Upper Bound
C. destructans	58.0	1.662	54.7	61.2
C. liriodendri	53.7	1.662	50.4	56.9
C. macrodidymum	59.6	1.662	56.3	62.9

D.1.2 Analysis of variance of the effect of HWT using different temperatures and times combinations on conidium germination of nine *Cylindrocarpon* isolates

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Isolate	45.16	8	5.64	2908.99	<0.001
HWT temp	1311.33	3	437.11	225231.33	<0.001
HWT time	874.22	2	437.11	225231.33	<0.001
Isolate x HWT temp	135.49	24	5.65	2908.99	<0.001
Isolate x HWT time	90.33	16	5.65	2908.99	<0.001
HWT temp x HWT time	2622.66	6	437.11	225231.33	<0.001
Isolate x HWT temp x HWT time	270.98	48	5.65	2908.99	<0.001
Error	0.419	216	0.002		
Species x HWT temp x HWT time	270.85	12	22.57	9341.49	<0.001
Error	0.696	288	0.002		

Species	Mean	Standard error	95% Confidence interval	
			Lower Bound	Upper Bound
C. destructans	98.3	.005	98.3	98.3
C. liriodendri	99.0	.005	99.0	99.0
C. macrodidymum	99.1	.005	99.1	99.1

D.1.3 Analysis of Variance of the effect of HWT on mycelium plugs of nine *Cylindrocarpon* isolates implanted into trunk pieces on disease incidence.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Species	8130.4	2	4065.3	15.55	<0.001
Species x isolate	2052.5	6	342.1	1.31	0.260
HWT temp	105653.3	3	35217.8	134.71	<0.001
Species x temp	7283.4	6	1213.9	4.64	<0.001
Species x control vs. HWT	4831.4	2	2415.7	9.24	<0.001
Species x HWT	2452.0	4	613	2.35	0.059
Species x isolate x temp	8711.4	18	484.0	1.85	0.029
Species x isolate x control vs. HWT	6669.2	6	1111.5	4.25	<0.001
Error	25879.6	99	261.4		

D.1.4 Analysis of Variance of the effect of HWT on mycelium plugs of nine *Cylindrocarpon* isolates implanted into different positions in the trunk pieces on disease incidence.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Temp	316959.9	3	105653.3	229.1	<0.001
Isolate	30903.6	8	3862.9	8.38	<0.001
Position	53484.6	2	26742.3	57.98	<0.001
Temp x isolate	47166.1	24	1965.3	4.26	<0.001
Temp x position	19841.0	6	3306.8	7.17	<0.001
Isolate x position	16107.0	16	1006.7	2.18	0.006
Temp x isolate x position	17527.7	48	365.2	0.79	0.836
Error	136976.2	297	461.2		

D.1.5 Mycelium growth (mm) for nine *Cylindrocarpon* isolates after HWT at temperatures of (Temp.) 40, 45, 47, 50 and 55°C for zero minutes (Min) for the untreated control or 5, 15 or 30 min.

Temp.	Isolate	Min.	Mycelium growth (mm)	*	Temp.	Isolate	Min.	Mycelium growth (mm)	*	Temp.	Isolate	Min.	Mycelium growth (mm)	*	Temp.	Isolate	Min.	Mycelium growth (mm)	*
40°C	2L	0	51.00	a	40°C	2L	5 min	54.00	a	40°C	1M	15 min	54.00	a	40°C	2L	30 min	55.00	a
40°C	1L	0	57.00	ab	40°C	1M	5 min	56.33	a	40°C	1L	15 min	56.00	a	40°C	3M	30 min	58.67	a
40°C	2M	0	61.00	ab	40°C	2M	5 min	58.00	a	40°C	2M	15 min	56.00	a	40°C	2M	30 min	60.33	a
40°C	3M	0	61.00	ab	40°C	3M	5 min	61.67	a	40°C	2L	15 min	57.00	a	40°C	1M	30 min	60.67	a
40°C	1M	0	63.00	ab	40°C	2D	5 min	65.67	ab	40°C	3M	15 min	58.67	a	40°C	2D	30 min	61.33	a
40°C	2D	0	67.00	b	40°C	1L	5 min	67.67	ab	40°C	2D	15 min	65.00	ab	40°C	1L	30 min	65.33	ab
40°C	1D	0	80.00	c	40°C	1D	5 min	80.00	b	40°C	1D	15 min	78.00	b	40°C	1D	30 min	78.33	ab
40°C	3D	0	80.00	c	40°C	3D	5 min	80.00	b	40°C	3D	15 min	78.67	b	40°C	3D	30 min	79.33	b
40°C	3L	0	80.00	c	40°C	3L	5 min	80.00	b	40°C	3L	15 min	80.00	b	40°C	3L	30 min	80.00	b
45°C	2L	0	51.00	a	45°C	1L	5 min	49.67	a	45°C	3M	15 min	16.33	a	45°C	1D	30 min	0.00	a
45°C	1L	0	57.00	ab	45°C	2L	5 min	50.00	ab	45°C	2L	15 min	41.00	b	45°C	2D	30 min	0.00	a
45°C	2M	0	61.00	ab	45°C	3M	5 min	52.67	ab	45°C	2D	15 min	44.33	b	45°C	3D	30 min	0.00	a
45°C	3M	0	61.00	ab	45°C	1M	5 min	56.33	ab	45°C	1D	15 min	44.67	b	45°C	1L	30 min	0.00	a
45°C	1M	0	63.00	ab	45°C	2M	5 min	56.67	ab	45°C	3D	15 min	48.00	bc	45°C	2L	30 min	0.00	a
45°C	2D	0	67.00	b	45°C	2D	5 min	65.67	bc	45°C	1L	15 min	49.33	bc	45°C	1M	30 min	0.00	a
45°C	1D	0	80.00	c	45°C	1D	5 min	75.00	c	45°C	2M	15 min	53.00	bc	45°C	2M	30 min	0.00	a
45°C	3D	0	80.00	c	45°C	3D	5 min	77.33	c	45°C	1M	15 min	62.67	cd	45°C	3M	30 min	0.00	a
45°C	3L	0	80.00	c	45°C	3L	5 min	78.00	c	45°C	3L	15 min	75.33	d	45°C	3L	30 min	24.67	b
47°C	3L	0	40.00	a	47°C	3L	5 min	38.33	a	47°C	2D	15 min	0.00	a	47°C	1D	30 min	0.00	a
47°C	3M	0	54.00	ab	47°C	2L	5 min	42.00	a	47°C	3D	15 min	0.00	a	47°C	2D	30 min	0.00	a
47°C	2L	0	56.00	ab	47°C	1L	5 min	45.33	a	47°C	1L	15 min	0.00	a	47°C	3D	30 min	0.00	a
47°C	2M	0	59.00	ab	47°C	2D	5 min	50.00	ab	47°C	2L	15 min	0.00	a	47°C	2L	30 min	0.00	a
47°C	1L	0	64.00	ab	47°C	3M	5 min	50.00	ab	47°C	3L	15 min	0.00	a	47°C	3L	30 min	0.00	a
47°C	2D	0	64.00	ab	47°C	2M	5 min	52.33	ab	47°C	1M	15 min	0.00	a	47°C	1M	30 min	0.00	a
47°C	1M	0	68.00	bc	47°C	1M	5 min	62.67	b	47°C	3M	15 min	15.00	a	47°C	2M	30 min	0.00	a
47°C	1D	0	79.33	c	47°C	1D	5 min	72.33	bc	47°C	2M	15 min	32.33	b	47°C	3M	30 min	0.00	a
47°C	3D	0	80.00	c	47°C	3D	5 min	80.00	c	47°C	1D	15 min	64.00	c	47°C	1L	30 min	10.33	b
50°C	2L	0	51.00	a	50°C	3M	5 min	0.00	a	50°C	1D	15 min	0.00	a	50°C	1D	30 min	0.00	a
50°C	1L	0	57.00	ab	50°C	2L	5 min	15.33	b	50°C	2D	15 min	0.00	a	50°C	2D	30 min	0.00	a
50°C	2M	0	61.00	ab	50°C	1D	5 min	19.33	b	50°C	3D	15 min	0.00	a	50°C	3D	30 min	0.00	a
50°C	3M	0	61.00	ab	50°C	1M	5 min	32.00	bc	50°C	1L	15 min	0.00	a	50°C	1L	30 min	0.00	a
50°C	1M	0	63.00	ab	50°C	2D	5 min	37.33	c	50°C	2L	15 min	0.00	a	50°C	2L	30 min	0.00	a
50°C	2D	0	67.00	bc	50°C	2M	5 min	46.33	cd	50°C	3L	15 min	0.00	a	50°C	1M	30 min	0.00	a
50°C	1D	0	80.00	c	50°C	1L	5 min	56.67	d	50°C	1M	15 min	0.00	a	50°C	2M	30 min	0.00	a
50°C	3D	0	80.00	c	50°C	3L	5 min	72.67	e	50°C	2M	15 min	0.00	a	50°C	3M	30 min	0.00	a
50°C	3L	0	80.00	c	50°C	3D	5 min	73.00	e	50°C	3M	15 min	0.00	a	50°C	3L	30 min	24.67	b
55°C	2D	0	50.00	a	55°C	1D	5 min	0.00	a	55°C	1D	15 min	0.00	a	55°C	1D	30 min	0.00	a
55°C	3M	0	50.00	a	55°C	2D	5 min	0.00	a	55°C	2D	15 min	0.00	a	55°C	2D	30 min	0.00	a
55°C	1L	0	51.00	a	55°C	1L	5 min	0.00	a	55°C	3D	15 min	0.00	a	55°C	3D	30 min	0.00	a
55°C	2L	0	52.00	a	55°C	3L	5 min	0.00	a	55°C	1L	15 min	0.00	a	55°C	1L	30 min	0.00	a
55°C	2M	0	55.00	ab	55°C	1M	5 min	0.00	a	55°C	2L	15 min	0.00	a	55°C	2L	30 min	0.00	a
55°C	1M	0	60.00	ab	55°C	2M	5 min	0.00	a	55°C	3L	15 min	0.00	a	55°C	3L	30 min	0.00	a
55°C	1D	0	68.00	bc	55°C	3M	5 min	0.00	a	55°C	1M	15 min	0.00	a	55°C	1M	30 min	0.00	a
55°C	3L	0	70.00	bc	55°C	2L	5 min	14.67	a	55°C	2M	15 min	0.00	a	55°C	2M	30 min	0.00	a
55°C	3D	0	79.00	c	55°C	3D	5 min	16.00	b	55°C	3M	15 min	0.00	a	55°C	3M	30 min	0.00	a

* Values followed by the same letter are not significantly different according to Fisher's protected LSD. The overall isolate x temperature x time effect was significant (P<0.001, LSD 15.15) (right hand column of each set of data for each temperature).

D.2 *In vivo* Hot Water Treatment experiment

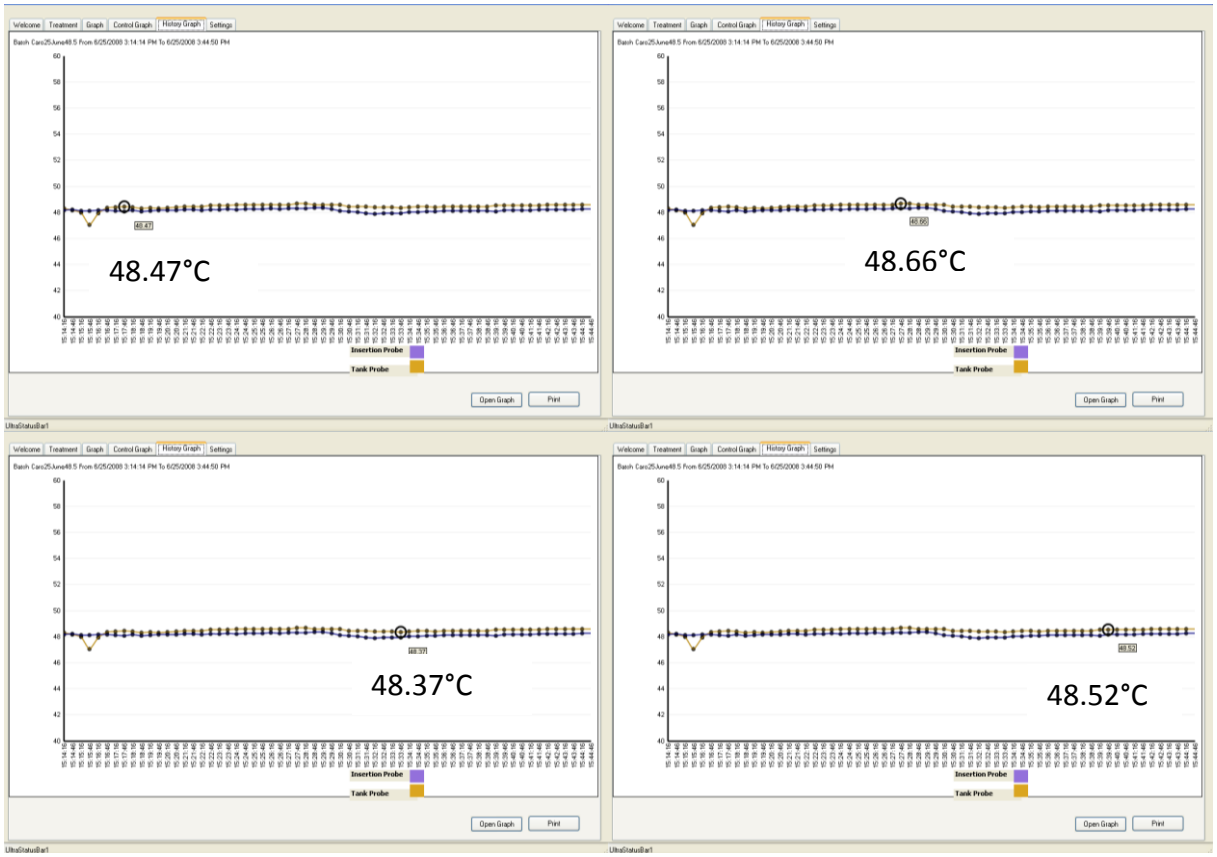
D.2.1 The effect of HWT on percent disease severity of grapevine rootstock varieties 101-14 and 5C which were planted into infested soil and then HWT at temperature/time combinations of 47, 48.5 and 50°C for 15 and 30 min.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	4155.04	6	692.51	20.51	0.001
Variety	9.42	1	9.42	0.28	0.599
HWT treatment x variety	54.87	6	9.15	0.27	0.949
Error	2194.59	65	33.76		

D.2.2 The effect of HWT on percent disease incidence of grapevine rootstock varieties 101-14 and 5C which were planted into infested soil and then HWT at temperature/time combinations of 47, 48.5 and 50°C for 15 and 30 min.

Variation	Sum of Squares	d.f	Mean Square	F ratio	P value
Treatment	19062.5	6	3177.08	40.11	0.001
Variety	119.05	1	119.05	1.50	0.225
HWT treatment x variety	401.79	6	66.96	0.85	0.540
Error	5148.81	65	79.21		

D.2.3 Computer readings of the HWT tank sensor probe (brown) and the portable insertion sensor probe (purple) used to monitor HWT (48.5°C for 30 min).



Appendix E

Statistical analysis and additional data for Chapter 4

E.1 Disease incidence

E.1.1 Analysis of Variance to test between subject effects for month of harvest, HWT, cold storage and variety on percent disease incidence (0 cm).

Disease Incidence (%)		df	Mean Square	F	Sig.
Intercept	Hypothesis	1	77824.074	265.771	.000
	Error	15	292.824[a]		
Month (mth)	Hypothesis	2	1860.532	6.354	.010
	Error	15	292.824[a]		
mth * Blocks	Hypothesis	15	292.824	.717	.755
	Error	45	408.565[b]		
HWT	Hypothesis	1	38935.185	95.297	.000
	Error	45	408.565[b]		
Cold Stored	Hypothesis	1	289.352	.708	.404
	Error	45	408.565[b]		
HWT * Cold Stored	Hypothesis	1	567.130	1.388	.245
	Error	45	408.565[b]		
mth * HWT	Hypothesis	2	1600.116	3.916	.027
	Error	45	408.565[b]		
mth * Cold Stored	Hypothesis	2	72.338	.177	.838
	Error	45	408.565[b]		
mth * HWT * Cold Stored	Hypothesis	2	436.921	1.069	.352
	Error	45	408.565[b]		
mth * HWT * Cold Stored * Blocks	Hypothesis	45	408.565	.994	.494
	Error	120	410.880[c]		
Variety	Hypothesis	2	3423.032	8.331	.000
	Error	120	410.880[c]		
mth * Variety	Hypothesis	4	245.949	.599	.664
	Error	120	410.880[c]		
HWT * Variety	Hypothesis	2	749.421	1.824	.166
	Error	120	410.880[c]		
Cold Stored * Variety	Hypothesis	2	298.032	.725	.486
	Error	120	410.880[c]		
HWT * Cold Stored * Variety	Hypothesis	2	20.255	.049	.952
	Error	120	410.880[c]		
mth *HWT * Cold Stored * Variety	Hypothesis	4	462.963	1.127	.347
	Error	120	410.880[c]		
mth * Cold Stored * Variety	Hypothesis	4	679.977	1.655	.165
	Error	120	410.880[c]		
mth * HWT * Variety	Hypothesis	4	393.519	.958	.433
	Error	120	410.880[c]		

a. MS (mth * Blocks)
b. MS (mth * HWT * Cold Stored * Blocks)
c. MS (Error)

E.2 Disease severity

E.2.1 Analysis of Variance to test between subject effects for month of harvest, HWT, cold storage and variety on percent disease severity.

Disease Severity %		df	Mean Square	F	Sig.
Intercept	Hypothesis	1	27225.116	277.756	.000
	Error	15	98.018[a]		
Month (mth)	Hypothesis	2	945.276	9.644	.002
	Error	15	98.018[a]		
mth * Blocks	Hypothesis	15	98.018	.668	.801
	Error	45	146.822[b]		
HWT	Hypothesis	1	13976.418	95.193	.000
	Error	45	146.822[b]		
Cold Stored	Hypothesis	1	35.446	.241	.626
	Error	45	146.822[b]		
HWT * Cold Stored	Hypothesis	1	416.667	2.838	.099
	Error	45	146.822[b]		
mth * HWT	Hypothesis	2	521.014	3.549	.037
	Error	45	146.822[b]		
mth * Cold Stored	Hypothesis	2	31.648	.216	.807
	Error	45	146.822[b]		
mth * HWT * Cold Stored	Hypothesis	2	134.006	.913	.409
	Error	45	146.822[b]		
mth * HWT * Cold Stored * Blocks	Hypothesis	45	146.822	.832	.756
	Error	120	176.505[c]		
Variety	Hypothesis	2	1922.924	10.894	.000
	Error	120	176.505[c]		
mth * Variety	Hypothesis	4	164.569	.932	.448
	Error	120	176.505[c]		
HWT * Variety	Hypothesis	2	733.688	4.157	.018
	Error	120	176.505[c]		
Cold Stored * Variety	Hypothesis	2	44.669	.253	.777
	Error	120	176.505[c]		
HWT * Cold Stored * Variety	Hypothesis	2	117.730	.667	.515
	Error	120	176.505 [c]		
mth *HWT * Cold Stored * Variety	Hypothesis	4	167.101	.947	.440
	Error	120	176.505[c]		
mth * Cold Stored * Variety	Hypothesis	4	252.459	1.430	.228
	Error	120			
mth * HWT * Variety	Hypothesis	4	87.529	.496	.739
	Error	120	176.505[c]		

a. MS (mth * Blocks)
b. MS (mth * HWT * Cold Stored * Blocks)
c. MS (Error)

E.3 Disease incidence 5 cm

E.3.1 Analysis of Variance to test between subject effects for month of harvest, HWT, cold storage and variety on percent disease incidence at 5 cm.

Disease incidence 5 cm		df	Mean Square	F	Sig.
Intercept	Hypothesis	1	15844.907	95.069	.000
	Error	15	166.667[a]		
Month (mth)	Hypothesis	2	46.296	.278	.761
	Error	15	166.667[a]		
mth * Blocks	Hypothesis	15	166.667	.675	.794
	Error	45	246.914[b]		
HWT	Hypothesis	1	9733.796	39.422	.000
	Error	45	246.914[b]		
Cold Stored	Hypothesis	1	11.574	.047	.830
	Error	45	246.914[b]		
HWT * Cold Stored	Hypothesis	1	567.130	2.297	.137
	Error	45	246.914[b]		
mth * HWT	Hypothesis	2	219.907	.891	.418
	Error	45	246.914[b]		
mth * Cold Stored	Hypothesis	2	358.796	1.453	.245
	Error	45	246.914[b]		
mth * HWT * Cold Stored	Hypothesis	2	324.074	1.312	.279
	Error	45	246.914[b]		
mth * HWT * Cold Stored * Blocks	Hypothesis	45	246.914	1.209	.208
	Error	120	204.282[c]		
Variety	Hypothesis	2	2190.394	10.722	.000
	Error	120	204.282[c]		
mth * Variety	Hypothesis	4	180.845	.885	.475
	Error	120	204.282[c]		
HWT * Variety	Hypothesis	2	1322.338	6.473	.002
	Error	120	204.282[c]		
Cold Stored * Variety	Hypothesis	2	89.699	.439	.646
	Error	120	204.282[c]		
HWT * Cold Stored * Variety	Hypothesis	2	124.421	.609	.546
	Error	120	204.282[c]		
mth *HWT * Cold Stored * Variety	Hypothesis	4	154.803	.758	.555
	Error	120	204.282[c]		
mth * Cold Stored * Variety	Hypothesis	4	215.567	1.055	.382
	Error	120	204.282[c]		
mth * HWT * Variety	Hypothesis	4	102.720	.503	.734
	Error	120	204.282[c]		

a. MS (mth * Blocks)
b. MS (mth * HWT * Cold Stored * Blocks)
c. MS (Error)

E.4 Water soluble carbohydrate sugar (%)

E.4.1 Analysis of Variance to test between subject effects for month of harvest, HWT, cold storage and variety on percent sugar.

WSC % Sugar		df	Mean Square	F	Sig.
Intercept	Hypothesis	1	16045.914	7592.867	.000
	Error	15	2.113[a]		
Month (mth)	Hypothesis	2	10.400	4.921	.023
	Error	15	2.113[a]		
mth * Blocks	Hypothesis	15	2.113	1.047	.429
	Error	45	2.019[b]		
HWT	Hypothesis	1	24.014	11.893	.001
	Error	45	2.019[b]		
Cold Stored	Hypothesis	1	441.092	218.455	.000
	Error	45	2.019[b]		
HWT * Cold Stored	Hypothesis	1	10.822	5.360	.025
	Error	45	2.019[b]		
mth * HWT	Hypothesis	2	.682	.338	.715
	Error	45	2.019[b]		
mth * Cold Stored	Hypothesis	2	22.751	11.268	.000
	Error	45	2.019[b]		
mth * HWT * Cold Stored	Hypothesis	2	6.933	3.434	.041
	Error	45	2.019[b]		
mth * HWT * Cold Stored * Blocks	Hypothesis	45	2.019	.995	.493
	Error	120	2.029[c]		
Variety	Hypothesis	2	19.883	9.801	.000
	Error	120	2.029[c]		
mth * Variety	Hypothesis	4	3.190	1.573	.186
	Error	120	2.029[c]		
HWT * Variety	Hypothesis	2	1.477	.728	.485
	Error	120	2.029[c]		
Cold Stored * Variety	Hypothesis	2	5.939	2.927	.057
	Error	120	2.029[c]		
HWT * Cold Stored * Variety	Hypothesis	2	.809	.399	.672
	Error	120	2.029[c]		
mth *HWT * Cold Stored * Variety	Hypothesis	4	.224	.110	.979
	Error	120	2.029[c]		
mth * Cold Stored * Variety	Hypothesis	4	.878	.433	.785
	Error	120	2.029[c]		
mth * HWT * Variety	Hypothesis	4	4.359	2.149	.079
	Error	120	2.029[c]		

a. MS (mth * Blocks)
b. MS (mth * HWT * Cold Stored * Blocks)
c. MS (Error)

E.5 Water soluble carbohydrate starch (%)

E.5.1 Analysis of Variance to test between subject effects for month of harvest, HWT, cold storage and variety on percent starch.

Starch %		df	Mean Square	F	Sig.
Intercept	Hypothesis	1	14814.748	3766.791	.000
	Error	15	3.933[a]		
Month (mth)	Hypothesis	2	132.360	33.654	.000
	Error	15	3.933[a]		
mth * Blocks	Hypothesis	15	3.933	1.623	.106
	Error	45	2.424[b]		
HWT	Hypothesis	1	10.277	4.240	.050
	Error	45	2.424[b]		
Cold Stored	Hypothesis	1	171.443	70.727	.000
	Error	45	2.424[b]		
HWT * Cold Stored	Hypothesis	1	.402	.166	.686
	Error	45	2.424[b]		
mth * HWT	Hypothesis	2	.390	.161	.852
	Error	45	2.424[b]		
mth * Cold Stored	Hypothesis	2	3.347	1.381	.262
	Error	45	2.424[b]		
mth * HWT * Cold Stored	Hypothesis	2	2.950	1.217	.306
	Error	45	2.424[b]		
mth * HWT * Cold Stored * Blocks	Hypothesis	45	2.424	.973	.529
	Error	120	2.492[c]		
Variety	Hypothesis	2	111.063	44.567	.000
	Error	120	2.492[c]		
mth * Variety	Hypothesis	4	.733	.294	.881
	Error	120	2.492[c]		
HWT * Variety	Hypothesis	2	1.300	.521	.595
	Error	120	2.492[c]		
Cold Stored * Variety	Hypothesis	2	5.396	2.165	.119
	Error	120	2.492[c]		
HWT * Cold Stored * Variety	Hypothesis	2	3.577	1.435	.242
	Error	120	2.492[c]		
mth *HWT * Cold Stored * Variety	Hypothesis	4	4.316	1.732	.147
	Error	120	2.492[c]		
mth * Cold Stored * Variety	Hypothesis	4	4.098	1.645	.167
	Error	120	2.492[c]		
mth * HWT * Variety	Hypothesis	4	6.115	2.454	.050
	Error	120	2.492[c]		

a. MS (mth * Blocks)
b. MS (mth * HWT * Cold Stored * Blocks)
c. MS (Error)

E.6 Growth stage to E-L 9

E.6.1 Analysis of Variance to test between subject effects for month of harvest, HWT, cold storage and variety on bud growth to E-L 9 (weeks).

Mean weeks to E-L 9		df	Mean Square	F	Sig.
Intercept	Hypothesis	1	8510.362	14291.057	.000
	Error	15	.596[a]		
Month (mth)	Hypothesis	2	39.722	66.703	.000
	Error	15	.596[a]		
mth * Blocks	Hypothesis	15	.596	1.513	.141
	Error	45	.394[b]		
HWT	Hypothesis	1	39.061	99.226	.000
	Error	45	.394[b]		
Cold Stored	Hypothesis	1	98.840	251.081	.000
	Error	45	.394[b]		
HWT * Cold Stored	Hypothesis	1	12.653	32.141	.000
	Error	45	.394[b]		
mth * HWT	Hypothesis	2	3.175	8.065	.001
	Error	45	.394[b]		
mth * Cold Stored	Hypothesis	2	41.767	106.099	.000
	Error	45	.394[b]		
mth * HWT * Cold Stored	Hypothesis	2	1.811	4.601	.015
	Error	45	.394[b]		
mth * HWT * Cold Stored * Blocks	Hypothesis	45	.394	1.449	.058
	Error	120	.272[c]		
Variety	Hypothesis	2	5.148	18.955	.000
	Error	120	.272[c]		
mth * Variety	Hypothesis	4	.570	2.099	.085
	Error	120	.272[c]		
HWT * Variety	Hypothesis	2	.009	.034	.967
	Error	120	.272[c]		
Cold Stored * Variety	Hypothesis	2	.674	2.481	.088
	Error	120	.272[c]		
HWT * Cold Stored * Variety	Hypothesis	2	.168	.620	.540
	Error	120	.272[c]		
mth *HWT * Cold Stored * Variety	Hypothesis	4	.052	.192	.942
	Error	120	.272[c]		
mth * Cold Stored * Variety	Hypothesis	4	.440	1.621	.173
	Error	120	.272[c]		
mth * HWT * Variety	Hypothesis	4	.066	.243	.914
	Error	120	.272[c]		

a. MS (mth * Blocks)
b. MS (mth * HWT * Cold Stored * Blocks)
c. MS (Error)

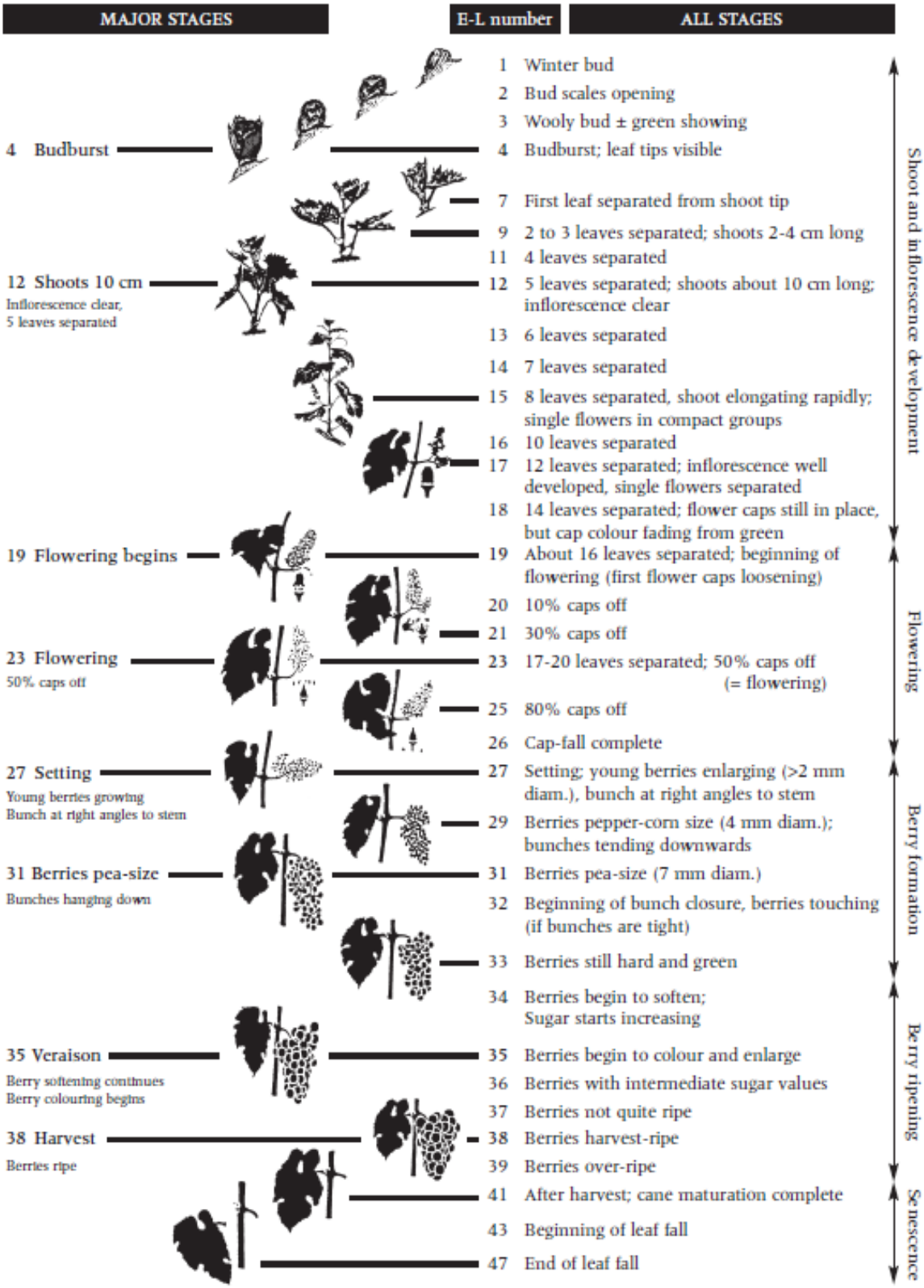
E.7 Growth stage to E-L 9

E.7.1 Analysis of Variance to test between subject effects for month of harvest, HWT, cold storage and variety on bud growth to E-L 4 (weeks).

Source		Sum of Squares	d.f.	Mean Square	F	Sig.
Intercept	Hypothesis	4887.912	1	4887.912	27635.390	.000
	Error	2.653	15	.177[a]		
mth	Hypothesis	68.595	2	34.297	193.911	.000
	Error	2.653	15	.177[a]		
mth*Blocks	Hypothesis	2.653	15	.177	1.370	.203
	Error	5.808	45	.129[b]		
HWT	Hypothesis	27.543	1	27.543	213.384	.000
	Error	5.808	45	.129[b]		
ColdStored	Hypothesis	70.295	1	70.295	544.595	.000
	Error	5.808	45	.129[b]		
HWT*ColdStored	Hypothesis	21.4987	1	21.498	166.553	.000
	Error	5.808	45	.129[b]		
mth*HWT	Hypothesis	14.012	2	7.006	54.278	.000
	Error	5.808	45	.129[b]		
mth*ColdStored	Hypothesis	52.429	2	26.214	203.090	.000
	Error	5.808	45	.129[b]		
mth*HWT*ColdStored	Hypothesis	6.188	2	3.094	23.970	.000
	Error	5.808	45	.129[b]		
mth*HTW*ColdStored *Blocks	Hypothesis	5.808	45	.129	.921	.614
	Error	16.810	120	.140[c]		
Variety	Hypothesis	5.529	2	2.765	19.736	.000
	Error	16.810	120	.140[c]		
mth.Variety	Hypothesis	2.046	4	.511	3.651	.008
	Error	16.810	120	.140[c]		
HTW*Variety	Hypothesis	1.632	2	.816	5.824	.004
	Error	16.810	120	.140[c]		
ColdStored*Variety	Hypothesis	2.143	2	1.071	7.648	.001
	Error	16.810	120	.140[c]		
HWT*ColdStored*Variety	Hypothesis	.528	2	.264	1.884	.156
	Error	16.810	120	.140[c]		
mth*HWT*ColdStored *Variety	Hypothesis	1.018	4	.255	1.817	.130
	Error	16.810	120	.140[c]		
mth*ColdStored*Variety	Hypothesis	.647	4	.162	1.155	.334
	Error	16.810	120	.140[c]		
mth*HWT*Variety	Hypothesis	.817	4	.204	1.458	.219
	Error	16.810	120	.140[c]		

[a]MS (mth*Blocks)
[b]MS (mth*HWT*Cold Stored*Blocks)
[c]MS (Error)

E.7.2 Modified E-L system for identifying grapevine growth stages (Coombe, 1995).



E.8 Carbohydrate Root Analysis Method

Adapted protocol for sugar content analysis in ryegrass tissues (Pollock and Jones, 1979) modified for sugar content analysis in roots of grafted grapevines (D Keaney nee Monson, pers. comm., 2009).

Materials and methods for micro-analysis

A. Extraction of low molecular weight (LMW) water soluble carbohydrates (WSC)

Weight approx. 25 mg dried tissues into 2mL screw cap tubes

Add 1 mL of 80% ethanol (EtOH) (v/v)

Shake 30 min at 65°C

Centrifuge 15 min at 13000 rpm

Pipette supernatant (liquid) into 2mL Eppendorf tube

Add another 1 mL of 80% EtOH to residue

Shake 30 min at 65°C

Centrifuge 15 min at 13000 rpm

Combine supernatants

Store at -20°C for analysis

B. Extraction of high molecular weight (HMW) water soluble carbohydrates

After process A described above:

Add 1 mL of deionised water to residue

Shake 30 min at 65°C

Centrifuge 15 min at 13000 rpm

Pipette supernatant into new 2 mL Eppendorf tube

Add another 1 mL of H_2O to residue

Shake 30 min at 65°C

Centrifuge 15 min at 13000 rpm

Combine supernatants

Store at -20°C for analysis

C. Anthrone Reagent

Cool 30 mL 100% EtOH on ice

Slowly add 50 mL conc. H_2SO_4 (heat is by-product so extreme caution)

Cool down to room temp

Add 100 mg anthrone and mix well (use within 24 h)

Add 200 mg anthrone to 160 ml H_2SO_4 + EtOH mixture

Reduce wastage by making up smaller quantity:

62.5 mg anthrone ⟶ 50 mL H_2SO_4 + EtOH

Sugar Analysis

Adapted protocol for sugar content analysis (Jermyn, 1956) for sugar content analysis in roots of grafted grapevines (D. Keaney nee Monson, pers. comm., 2009).

D. For LMW carbohydrates

Take 12 µL extracts into a microwell and add 188 µL of water for master diluted samples

(3 µL/assay x 4 = 12ul)

Mix and remove 40 µL of diluted extract to a new 96 microwell plate

This microwell plate will hold 24 samples (72 wells + 24 wells containing standards)

Make 3 replicates of each

Use sucrose standards at conc. of 0, 10, 20, 30, 40, 50, 75 & 100 µL/mL

Again 3 reps

Add 200 µL of anthrone reagent

Shake and incubate at 65°C for 25 min

Shake 5 seconds and read absorbance at 620 nm 620 nm. (Automatically done by FLUOstar)

If using round bottom microwell plate remove 40 µL extract and 200 µL of anthrone reagent

E. For HMW carbohydrates

Take 40 µL extracts into a microwell and add 160 µL of water for master diluted samples

Mix and remove 40 µL of diluted extract to a new microwell

Use insulin standards at conc. of 0, 10, 20, 30, 40, 50, 75 & 100 µL/ml

Make 3 replicates of each

Add 200 µL of anthrone

Shake and incubate at 65°C for 25 min

Shake 5 s and read absorbance at 620 nm (automatically done by FLUOstar)

Notes: standards give absorbance curves up to 1.0 and it is expected that a straight line exists to 2.0; sample absorbance values greater than 2.0 should be repeated at higher dilution rates.

To prepare standards pre-mix a larger quantity at 100 µg/mL (e.g. stock solution 10 mg/10 mL) and store at -20C

Starch Analysis

G. Extraction of Total Starch

Based on modified Megazyme Assay Procedure K-TSTA 04/2009, (Megazyme, International, Bray, Ireland) (D. Keaney nee Monson, pers. comm., 2009)

1
- Add 0.3 mL dimethyl sulfoxide (DMSO) to the residue after HMW extraction
- Vortex, place tube in a boiling water bath (5 min), stir the tube after 2 and 4 min
- *Megazyme total starch procedure Page 11 (d) 4.*
- Immediately add 0.65 mL of thermostable α-amylase (Bottle 1) (300 U) in **MOPS buffer (50 mM, pH 7.0)

Vortex, incubate in a boiling water bath (6 min), stir after 2 and 4 min.

Place tube in a heating block (50^{o}C) to cool down solution

Soluble starch

2
- Add 0.7 mL sodium acetate buffer (200 mM, pH 4.5)
- Add amyloglucosidase (Bottle 2) (20 µL, 0.5 U)
- Vortex, incubate (50^{o}C, 30 min)
- Centrifuge (13,000 rpm, 15 min)

Pipette supernatant into 1.5 mL Eppendorf tube for assay

Insoluble starch

3
- Add *9 µL of starch extracts into wells of a 96 well plate
- React with *270 µL GOPOD reagent (Bottle 3 + 4) at 50^{o}C, 20 min
- Read at 510 nm

Use glucose solutions as calibration standards.

*quantity adjusted to fit into 300 µL microwell plates

GOPOD reagent: (Bottle 3 + 4) reagent from Megazyme (contains glucose oxidase, peroxidase, 4-aminoantipyrine and buffer) make up according to the manufacturer's instructions.

Standards and controls:

Control - Maize: (Bottle 6) from Megazyme kit: diluted 1:900

Standards: (Bottle 5)

Blank	1000 µL H_2O
100 µL D-Glucose +	900 µL H_2O (9 µL)
200 µL D-Glucose +	800 µL H_2O (9 µL)

500 µL D-Glucose + 500 µL H_2O (9 µL)

1000 µL D-Glucose (9 µL)

Soluble starch and glucose solutions (0.1, 0.2, 0.5, 1.0 mg/mL each) 9 µL solution/assay

**MOPS buffer (50 mM, pH 7.0) plus calcium chloride (5 mM) and sodium azide (0.02% w/v). Dissolve 11.55 g of MOPS (sodium salt, Sigma cat. No. M-9381) in 900 mL of distilled water and adjust to pH 7.0 by the addition of 1 M (10% v/v) HCl (approx. 17 mL is required). Add 0.74 g of calcium chloride dehydrate and 0.2 g sodium azide and dissolve. Adjust to 1 L. Stable for 6 months at 4°C (Megazyme, 2009).

E.9 Starch spread sheet example

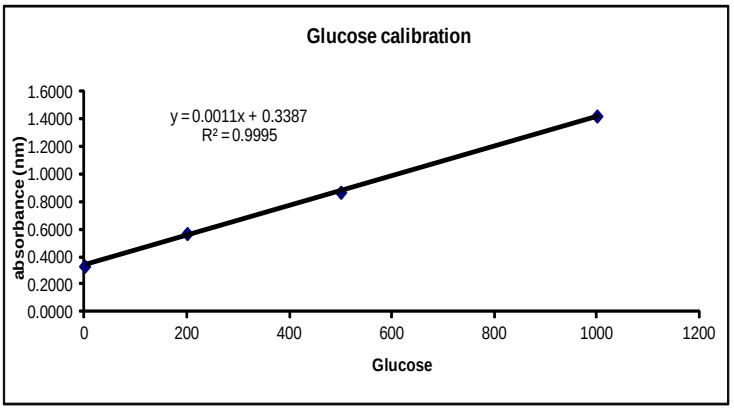
Calculations:	% STARCH
0.9	Hydrolysis factor (Adjustment from free D-glucose to anhydro D-glucose - as occurs in starch i.e. 162/180 (-1 H_2O)
x=(y-0.3387)/0.0011	Glucose calibration curve equation. **Note: This eq. changes each time a new plate is scanned**
1.67	Dilution Factor

Sample ID	ABSa	ABSb	ABSc	ABSmean	ug/ml	ug total	Sample Wgt / mg	mg / mg Starch	Average	% Diff	% Starch
1	1.094	1.033	1.086	1.07100	665.73	1111.76	25.2	0.04	0.043	-15.50	79.41
2	1.109	1.185	1.278	1.19067	774.52	1293.44	25.1	0.05			92.76
3	2.68	2.483	2.839	2.66733	2116.94	3535.29	25	0.13	0.126	2.51	12.73
4	2.665	2.461	2.703	2.60967	2064.52	3447.74	25	0.12			12.41
5	1.178	1.208	1.328	1.23800	817.55	1365.30	25	0.05	0.052	-12.48	4.92
6	1.397	1.275	1.401	1.35767	926.33	1546.98	25	0.06			5.57
7	1.411	1.455	1.507	1.45767	1017.24	1698.79	25	0.06	0.061	0.99	6.12
8	1.399	1.497	1.444	1.44667	1007.24	1682.09	25	0.06			6.06
9	1.816	2.211	1.75	1.92567	1442.70	2409.30	25	0.09	0.084	5.95	8.67
10	1.779	1.786	1.937	1.83400	1359.36	2270.14	25	0.08			8.17
11	1.387	1.436	1.305	1.37600	943.00	1574.81	25	0.06	0.061	-12.84	5.67
12	1.455	1.551	1.549	1.51833	1072.39	1790.90	25	0.06			6.45
13	1.674	1.909	1.901	1.82800	1353.91	2261.03	25	0.08	0.079	5.99	8.14
14	1.844	1.621	1.759	1.74133	1275.12	2129.45	25	0.08			7.67
15	2.962	2.693	2.451	2.70200	2148.45	3587.92	25	0.13	0.130	-1.98	12.92
16	2.457	2.865	2.926	2.74933	2191.48	3659.78	25	0.13			13.18
17	1.489	1.536	2.382	1.80233	1330.58	2222.06	25	0.08	0.075	11.97	8.00
18	1.691	1.636	1.584	1.63700	1180.27	1971.06	25	0.07			7.10
19	2.266	2.525	2.966	2.58567	2042.70	3411.30	25	0.12	0.122	2.14	12.28
20	2.599	2.484	2.531	2.53800	1999.36	3338.94	25	0.12			12.02
21	2.5	2.822	3.098	2.80667	2243.61	3746.82	25	0.13	0.133	2.14	13.49
22	2.759	2.709	2.795	2.75433	2196.03	3667.37	25	0.13			13.20
23	2.443	2.517	2.348	2.43600	1906.64	3184.08	25	0.11	0.115	-1.25	11.46
24	2.479	2.425	2.483	2.46233	1930.58	3224.06	25	0.12			11.61

% Starch in maize control (x Dil Factor of 20)

mg/ml	(mg/ml)	Glucose STD Average
0	0	0.3325
0.2	200	0.5690
0.5	500	0.8687
1	1000	1.4213

Glucose STD ABS			Average
	0.319	0.346	0.3325
0.473	0.509	0.434	0.4720
0.573	0.549	0.585	0.5690
0.846	0.867	0.893	0.8687
1.442	1.396	1.426	1.4213



E.10 Sugar spread sheet example

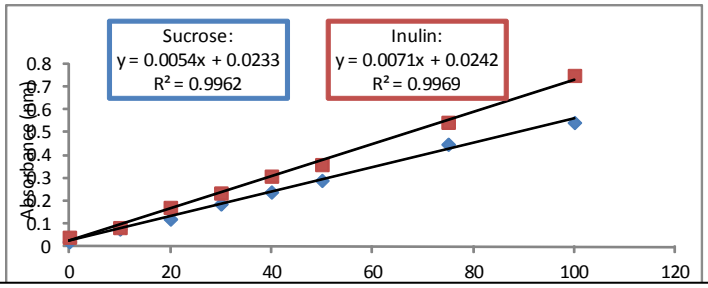
2 mL total extracts
Sucrose calibration curve equation: x=(y-0.0233)/0.00

Sample ID	ABSa	ABSb	ABSc	ABSmean	LMW ug/assay	LMW ug total	mg DW	mg LMW/ g DW	Average	% Diff	Sugar %CHO
1	0.301	0.2596	0.2757	0.27877	47.31	1576.95	24.6	64.10	64.31	0.63	6.41
2	0.301	0.2757	0.2676	0.28143	47.80	1593.42	24.7	64.51			6.45
3	0.6021	0.5528	0.6021	0.58567	104.14	3471.40	24.5	141.69	147.48	7.85	14.17
4	0.6778	0.6383	0.6383	0.65147	116.33	3877.57	25.3	153.26			15.33
5	0.6576	0.6021	0.6383	0.63267	112.85	3761.52	25.3	148.68	147.90	1.05	14.87
6	0.6198	0.585	0.6383	0.61437	109.46	3648.56	24.8	147.12			14.71
7	0.3372	0.3098	0.301	0.31600	54.20	1806.79	24.7	73.15	77.62	11.51	7.31
8	0.3872	0.3468	0.3372	0.35707	61.81	2060.29	25.1	82.08			8.21
9	0.2218	0.2366	0.2007	0.21970	36.37	1212.35	24.5	49.48	49.65	0.67	4.95
10	0.2291	0.2218	0.2291	0.22667	37.66	1255.35	25.2	49.82			4.98
11	0.1308	0.1308	0.1427	0.13477	20.64	688.07	25.9	26.57	27.32	5.53	2.66
12	0.1367	0.1367	0.1308	0.13473	20.64	687.86	24.5	28.08			2.81
13	0.2147	0.2291	0.2291	0.22430	37.22	1240.74	25	49.63	49.17	1.88	4.96
14	0.2291	0.2007	0.2366	0.22213	36.82	1227.37	25.2	48.71			4.87
15	0.1871	0.1367	0.1612	0.16167	25.62	854.12	24.5	34.86	34.41	2.61	3.49
16	0.1549	0.1612	0.1549	0.15700	24.76	825.31	24.3	33.96			3.40
17	0.1308	0.1549	0.1308	0.13883	21.40	713.17	24.6	28.99	28.88	0.78	2.90
18	0.1487	0.1308	0.1427	0.14073	21.75	724.90	25.2	28.77			2.88
19	0.2218	0.1739	0.1938	0.19650	32.07	1069.14	24.7	43.28	42.94	1.61	4.33
20	0.1805	0.1938	0.2007	0.19167	31.18	1039.30	24.4	42.59			4.26
21	0.1249	0.1249	0.1192	0.12300	18.46	615.43	24	25.64	24.11	12.69	2.56
22	0.1192	0.0969	0.1249	0.11367	16.73	557.82	24.7	22.58			2.26
23	0.1249	0.1192	0.1192	0.12110	18.11	603.70	24.8	24.34	28.50	29.15	2.43
24	0.1487	0.1487	0.1549	0.15077	23.60	786.83	24.1	32.65			3.26

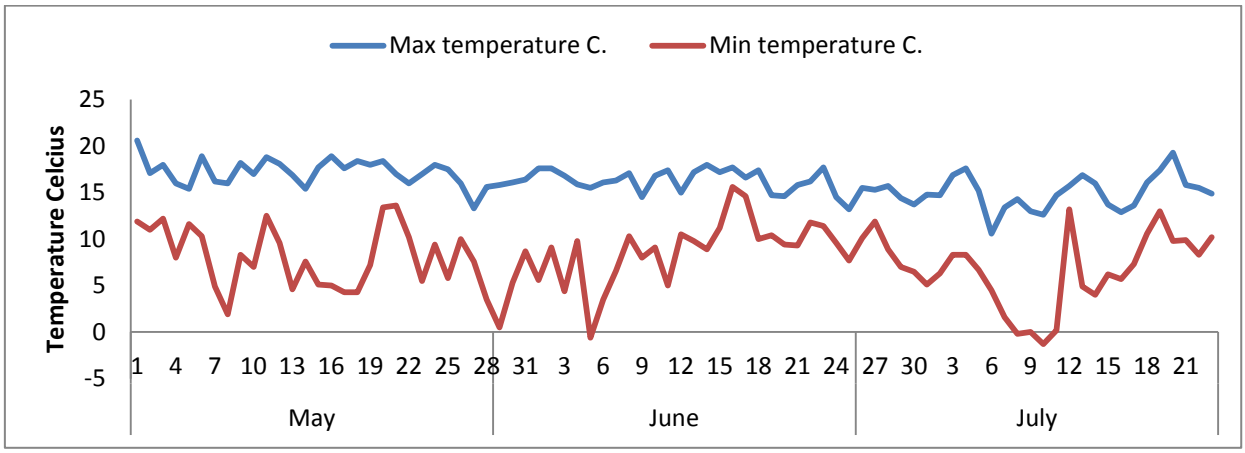
2 mL total extracts
Inulin calibration curve equation: x=(y-0.0242)/0.0071

Sample ID	ABSa	ABSb	ABSc	ABSmean	HMW ug/ml	HMW ug total	mg DW	mg HMW/ g DW	Average	% Diff	%CHO	Total WSC mg/g DW	%CHO
1	0.3054	0.2967	0.3235	0.309	40.05	400.47	24.6	16.28	15.91	4.58	1.63	80.38	8.04
2	0.2967	0.2796	0.3144	0.297	38.41	384.08	24.7	15.55			1.55	80.06	8.01
3	0.9956	0.9956	1.0414	1.011	138.97	1389.67	24.5	56.72	56.25	1.68	5.67	198.41	19.84
4	1.0414	0.9956	1.0414	1.026	141.12	1411.17	25.3	55.78			5.58	209.04	20.90
5	1.0414	0.9542	1.0414	1.012	139.17	1391.74	25.3	55.01	55.52	1.85	5.50	203.69	20.37
6	1.0414	0.9956	0.9956	1.011	138.97	1389.67	24.8	56.04			5.60	203.15	20.32
7	0.1505	0.1761	0.1568	0.161	19.29	192.86	24.7	7.81	8.86	23.66	0.78	80.96	8.10
8	0.2474	0.2103	0.1444	0.201	24.86	248.59	25.1	9.90			0.99	91.99	9.20
9	0.0714	0.0714	0.0766	0.073	6.89	68.92	24.5	2.81	3.07	16.75	0.28	52.30	5.23
10	0.0714	0.0818	0.098	0.084	8.38	83.85	25.2	3.33			0.33	53.14	5.31
11	0.0925	0.1091	0.1383	0.113	12.55	125.49	25.9	4.85	6.39	48.24	0.48	31.41	3.14
12	0.1206	0.1761	0.1895	0.162	19.42	194.18	24.5	7.93			0.79	36.00	3.60
13	0.1148	0.1444	0.1631	0.141	16.42	164.18	25	6.57	6.66	2.91	0.66	56.20	5.62
14	0.1264	0.1264	0.1827	0.145	17.04	170.38	25.2	6.76			0.68	55.47	5.55
15	0.1761	0.2032	0.2248	0.201	24.95	249.53	24.5	10.18	11.36	20.61	1.02	45.05	4.50
16	0.2103	0.2632	0.2474	0.240	30.44	304.37	24.3	12.53			1.25	46.49	4.65
17	0.0872	0.1148	0.0925	0.098	10.42	104.18	24.6	4.23	4.34	4.68	0.42	33.23	3.32
18	0.1035	0.0925	0.1148	0.104	11.18	111.83	25.2	4.44			0.44	33.20	3.32
19	0.0925	0.0872	0.1264	0.102	10.96	109.62	24.7	4.44	4.89	18.50	0.44	47.72	4.77
20	0.1148	0.1264	0.1091	0.117	13.04	130.38	24.4	5.34			0.53	47.94	4.79
21	0.1323	0.1568	0.1505	0.147	17.23	172.30	24	7.18	5.80	47.63	0.72	32.82	3.28
22	0.1035	0.1035	0.098	0.102	10.91	109.11	24.7	4.42			0.44	27.00	2.70
23	0.1383	0.1206	0.098	0.119	13.35	133.47	24.8	5.38	6.52	34.78	0.54	29.72	2.97
24	0.1568	0.1323	0.1761	0.155	18.43	184.32	24.1	7.65			0.76	40.30	4.03

(mg/ml)	S Average	I Average
0	0.0223	0.0415
10	0.0774	0.0837
20	0.1211	0.1720
30	0.1871	0.2348
40	0.2391	0.3085
50	0.2898	0.3589
75	0.4479	0.5438
100	0.5430	0.7487



E.10.1 May, June and July daily maximum and minimum temperatures (°C) for 28 days prior to harvest of grafted grapevines from the Auckland HWT experiment (2008).



Appendix F

Statistical analysis for Chapter 5

F.1 *In vivo* preliminary biofumigation experiment.

F.1.1 Analysis of Variance to test effect of treatments on disease incidence.

Variation Incidence 2007 Biofumigation (%)	d.f.	Sum of Squares	Mean Square	F. Ratio	P. Value
Treatment	3	1324.2	441.4	1.16	0.338
Variety	1	660.2	660.2	1.74	0.196
Treatment.Variety	3	1793.0	597.7	1.58	0.214
Residual	32	12125.0	378.9		

F.1.2 Analysis of Variance to test effect of treatments on disease severity.

Variation Severity (%)	d.f.	Sum of Squares	Mean Square	F. Ratio	P. Value
Treatment	3	604.2	201.4	1.38	0.267
Variety	1	41.3	41.3	0.28	0.599
Treatment.Variety	3	565.2	188.4	1.29	0.295
Residual	32	4675.8	146.1		

F.1.3 Analysis of Variance to test effect of treatments on disease incidence (5 cm).

Variation 5cm (%)	d.f.	Sum of Squares	Mean Square	F. Ratio	P. Value
Treatment	3	761.7	253.9	0.74	0.537
Variety	1	1128.9	1128.9	3.28	0.079
Treatment.Variety	3	668.0	222.7	0.65	0.590
Residual	32	11000.0	343.8		

F.2 *In vitro* biofumigation experiments – Mycelium

F.2.1 Analysis of Variance to test effect of treatments on mycelium growth (mm) of nine *Cylindrocarpon* isolates.

Variation Mycelium Growth	Sum of Squares	d.f.	Mean Square	F. Ratio	P.value
Corrected Model	13367.605[a]	53	252.219	19.225	.000
Intercept	106156.495	1	106156.495	8091.570	.000
Isolate	6527.231	8	815.904	62.191	.000
Treatment	4731.918	5	946.384	72.136	.000
Isolate.Treatment	2108.457	40	52.711	4.018	.000
Error	2833.789	216	13.119		
Total	122357.889	270			
Corrected Total	16201.394	269			

F.2.2 Analysis of Variance to test effect of treatments on mycelium growth (mm) of three *Cylindrocarpon* spp.

Species	Mean	Standard error	95% Confidence interval	
			Lower Bound	Upper Bound
C. destructans	25.5	0.505	24.5	26.5
C. liriodendri	18.4	0.505	17.4	19.4
C. macrodidymum	15.4	0.505	14.4	16.4

F.2.3 Analysis of Variance to test effect of treatments of three *Cylindrocarpon* spp. x treatment interaction on mycelium growth (mm).

		Mean	Std. Error	95% Confidence Interval	
Treatment	SPECIES			Lower Bound	Upper Bound
Control ME Agar	D	28.979	1.236	26.545	31.414
	L	19.61	1.236	17.175	22.045
	M	16.959	1.236	14.525	19.394
Mustard meal	D	24.473	1.236	22.039	26.908
	L	11.117	1.236	8.683	13.552
	M	11.581	1.236	9.147	14.016
Root and shoots	D	31.799	1.236	29.364	34.233
	L	26.803	1.236	24.368	29.237
	M	22.008	1.236	19.574	24.443
Soil + Mustard meal	D	24.26	1.236	22.025	26.895
	L	16.859	1.236	14.424	19.293
	M	11.268	1.236	8.833	13.703
Soil + Roots and shoots	D	17.042	1.236	14.607	19.47
	L	14.659	1.236	12.225	17.094
	M	13.018	1.236	10.583	15.453
Soil Control	D	26.757	1.236	24.322	29.191
	L	21.806	1.236	19.371	24.241
	M	17.715	1.236	15.281	20.15
LSD		3.42			

F.3 *In vitro* biofumigation experiments – Colony numbers

F.3.1 Analysis of Variance to test effect of treatments on conidia colony number of nine *Cylindrocarpon* isolates.

Variation Mycelium Growth	Sum of Squares	d.f.	Mean Square	F. Ratio	P.value
Corrected Model	57960.152	53	1093.588		.000
Intercept	123178.848	1	123178.848		.000
Treatment	16666.596	5	3333.319		.000
Isolate	33915.252	8	4239.406		.000
Treatment.Isolate	7378.304	40	184.458		.000
Error	7514.00	216	34.787		
Total	188653.00	270			
Corrected Total	65474.152	269			

F.3.2 Analysis of Variance to test effect of treatments on colony number of three *Cylindrocarpon* spp.

Species	Mean	Standard error	95% Confidence interval	
			Lower Bound	Upper Bound
C. destructans	29.7	1.208	27.365	32.124
C. liriodendri	20.6	1.208	18.287	23.047
C. macrodidymum	13.6	1.208	11.287	16.047

P<0.001, LSD=3.35

F.3.3 Analysis of Variance to test effect of treatments of *Cylindrocarpon* spp. x treatment interaction on colony number of three *Cylindrocarpon* spp.

		Mean	Std. Error	95% Confidence Interval	
Treatment	SPECIES			Lower Bound	Upper Bound
Control ME Agar	D	29	2.96	23.17	34.83
	L	24.733	2.96	18.904	30.563
	M	18.733	2.96	12.904	24.563
Mustard meal	D	4.933	2.96	-0.896	10.763
	L	4.933	2.96	-0.896	10.763
	M	2.6	2.96	-3.23	8.43
Root and shoots	D	36.467	2.96	30.637	42.296
	L	30.267	2.96	24.437	36.096
	M	16.467	2.96	10.637	22.296
Soil + Mustard meal	D	39.733	2.96	33.904	45.563
	L	20.533	2.96	14.704	26.363
	M	12.067	2.96	6.237	17.896
Soil + Roots and shoots	D	35.733	2.96	29.904	41.563
	L	20.067	2.96	14.237	25.896
	M	11.733	2.96	5.904	17.563
Soil Control	D	32.6	2.96	26.77	38.43
	L	23.467	2.96	17.637	29.296
	M	20.4	2.96	14.57	26.23
LSD		8.20			

F.4 *In vitro* biofumigation experiments – Colony size (mm)

F.4.1 Analysis of variance to test effect of treatments on colony size (mm) from conidia of nine *Cylindrocarpon* isolates.

Variation Average Colony Size (mm)	Sum of Squares	d.f.	Mean Square	F. Ratio	P.value
Corrected Model	4729.747[a]	53	89.241	53.096	.000
Intercept	8677.754	1	8677.754	5163.017	.000
Isolate	422.026	8	52.753	31.387	.000
Treatment	3995.037	5	799.007	475.387	.000
Isolate.Treatment	312.683	40	7.817	4.651	.000
Error	363.043	216	1.681		
Total	13770.543	270			
Corrected Total	5092.789	269			

[a]R. Squared = .929 (Adjusted R. Squared = .911)

F.4.2 Analysis of Variance to teat effect of treatments on colony size (mm) of three *Cylindrocarpon* spp.

Species	Mean	Standard error	95% Confidence interval	
			Lower Bound	Upper Bound
C. destructans	6.004	0.165	5.68	6.328
C. liriodendri	6.882	0.165	6.558	7.206
C. macrodidymum	4.122	0.165	3.798	4.446

P<0.001, LSD=0.46

F.4.3 Analysis of Variance to test effect of treatments of *Cylindrocarpon* spp. x treatment interaction on colony size (mm) of three *Cylindrocarpon* spp.

Treatment	**SPECIES**	**Mean**	**Std. Error**	**95% Confidence Interval**	
				Lower Bound	**Upper Bound**
Control ME Agar	D	10.566	0.403	9.772	11.36
	L	11.028	0.403	10.234	11.821
	M	7.733	0.403	6.94	8.527
Mustard meal	D	0.696	0.403	-9.77E-02	1.49
	L	1.975	0.403	1.181	2.768
	M	0.923	0.403	0.129	1.716
Root and shoots	D	2.293	0.403	1.5	3.087
	L	2.237	0.403	1.444	3.031
	M	1.425	0.403	0.632	2.219
Soil + Mustard meal	D	3.781	0.403	2.988	4.575
	L	5.004	0.403	4.21	5.798
	M	1.345	0.403	0.552	2.139
Soil + Roots and shoots	D	7.212	0.403	6.418	8.006
	L	8.331	0.403	7.537	9.124
	M	3.331	0.403	2.537	4.124
Soil Control	D	11.475	0.403	10.681	12.268
	L	12.719	0.403	11.925	13.512
	M	9.972	0.403	9.178	10.766
LSD		1.12			

F.5 *In vivo* biofumigation experiments

F.5.1 Analysis of Variance to test effect of treatments on disease severity.

Variation: Disease Severity		Sum of Squares	d.f.	Mean Square	F. Ratio	P.value.
Intercept	Hypothesis	2722.500	1	2722.500	103.714	.000
	Error	420.000	16	26.250[a]		
Treatment	Hypothesis	72.500	3	24.167	.921	.453
	Error	420.000	16	26.250[a]		
Treatment	Hypothesis	420.000	16	26.250	2.152	.068
*Block	Error	195.200	16	12.200[b]		
Variety	Hypothesis	864.900	1	864.900	70.893	.000
	Error	195.200	16	12.200[b]		
Treatment	Hypothesis	64.900	3	21.633	1.773	.193
*Variety	Error	195.200	16	12.200[b]		

[a]MS (Treatment*Block)
[b]MS (Error)

F.5.2 Analysis of Variance to test effect of treatments on disease incidence.

Variation: Disease incidence		Sum of Squares	d.f.	Mean Square	F. Ratio	P.value
Intercept	Hypothesis	504.100	1	504.100	118.264	.000
	Error	68.200	16	4.623[a]		
Treatment	Hypothesis	14.700	3	4.900	1.150	.359
	Error	68.200	16	4.263[a]		
Treatment	Hypothesis	68.200	16	4.263	3.376	.010
*Block	Error	20.200	16	1.263[b]		
Variety	Hypothesis	62.500	1	62.500	49.505	.000
	Error	20.200	16	1.263[b]		
Treatment	Hypothesis	4.300	3	1.433	1.135	.365
*Variety	Error	20.200	16	1.263[b]		

[a]MS (Treatment*Block)
[b]MS (Error)

F.5.3 Analysis of Variance to test effect of treatments on disease incidence (5 cm).

Variation: Disease incidence		Sum of Squares	d.f.	Mean Square	F. Ratio	P.value
Intercept	Hypothesis	140.625	1	140.625	80.357	.000
	Error	28.000	16	1.750[a]		
Treatment	Hypothesis	1.875	3	.625	.357	.785
	Error	28.000	16	1.750[a]		
Treatment *Block	Hypothesis	28.000	16	1.750	1.167	.381
	Error	24.000	16	1.500[b]		
Variety	Hypothesis	30.625	1	30.625	20.417	.000
	Error	24.000	16	1.500[b]		
Treatment *Variety	Hypothesis	3.875	3	1.292	.861	.481
	Error	24.000	16	1.500[b]		

[a]MS (Treatment*Block)
[b]MS (Error)

F.5.4 Analysis of Variance to test effect of treatments on disease severity.

Variation: Disease severity Dunnett t (2-sided) [a]		Sum of Squares	d.f.	Mean Square	F. Ratio	P.value
Intercept	Hypothesis	1361.2	1	1361.2	34.988	.004
Error		155.6	4	38.9		
Treatment	Hypothesis	36.2	3	12.0	2.667	0.095
Error		54.3	12	4.5		
Treatment	Hypothesis	155.6	4	38.9	8.586	0.002
Error		54.3	12	4.5		

Based on observed means.

[a]Dunnett t-tests treat one group as a control and compare all other groups against it.